Vol. 91 Part 3 September 1950

The Quarterly Journal of Microscopical Science

(Third Series, No. 14)

JOINT EDITORS

C. F. A. PANTIN, Sc.D., F.R.S.
JOHN R. BAKER, D.Sc.

ASSISTED BY

WILLIAM HOLMES, D.Phil.

Published for the Company of Biologists Limited

OXFORD: AT THE CLARENDON PRESS
LONDON: GEOFFREY CUMBERLEGE

PRINTED IN GREAT BRITAIN
BY CHARLES BATEY AT THE UNIVERSITY PRESS, OXFORD

Entered as second class at the New York, U.S.A., Post Office

Price 21*s*. net Subscription per vol. £3. 3*s*.

Volume 91 Part 3

CONTENTS

E. R. Trueman. Observations on the Ligament of *Mytilus edulis*. 22

B. Morris. The Structure of the Foetal Yolk-sac Splanchnopleur of the Rabbit. 23

A. F. W. Hughes. The Effect of Inhibitory Substances on Cell Division. With four Plates. 25

Y. T. Chen. Investigations into the Biology of *Peranema trichophorum* (Euglenineae). 27

L. Lison and **W. Mutsaars.** Metachromasy of Nucleic Acids. 30

Frances Macdonald. An Investigation of the Histochemical Technique for the Localization of Acid Phosphomonoesterase. With Plate. 31

C. H. Brown. A Review of the Methods available for the Determination of the Types of Forces Stabilizing Structural Proteins in Animals. 33

Emmy Klieneberger-Nobel. Methods for the Study of the Cytology of Bacteria and Pleuropneumonia-like Organisms. With Plate. 34

A. G. Oettlé. On the Use of Wide Illuminating Cones in the Microscope. 34

BOOKS ON THE PURE AND APPLIED SCIENCES always in stock

FOREIGN BOOKS. Select stock available. Those not in stock obtained to order under Board of Trade licence at the most favourable rates. Catalogues on request.

Please state interests

SCIENTIFIC LENDING LIBRARY

Annual Subscription from ONE GUINEA

Prospectus post free on application

The Library includes all recent and standard works in all branches of Science, and is particularly useful to Societies, Institutions, and Schools. Bi-monthly list of New Books and New Editions sent post free to subscribers on request

SECONDHAND BOOK DEPT.: 140 GOWER ST.

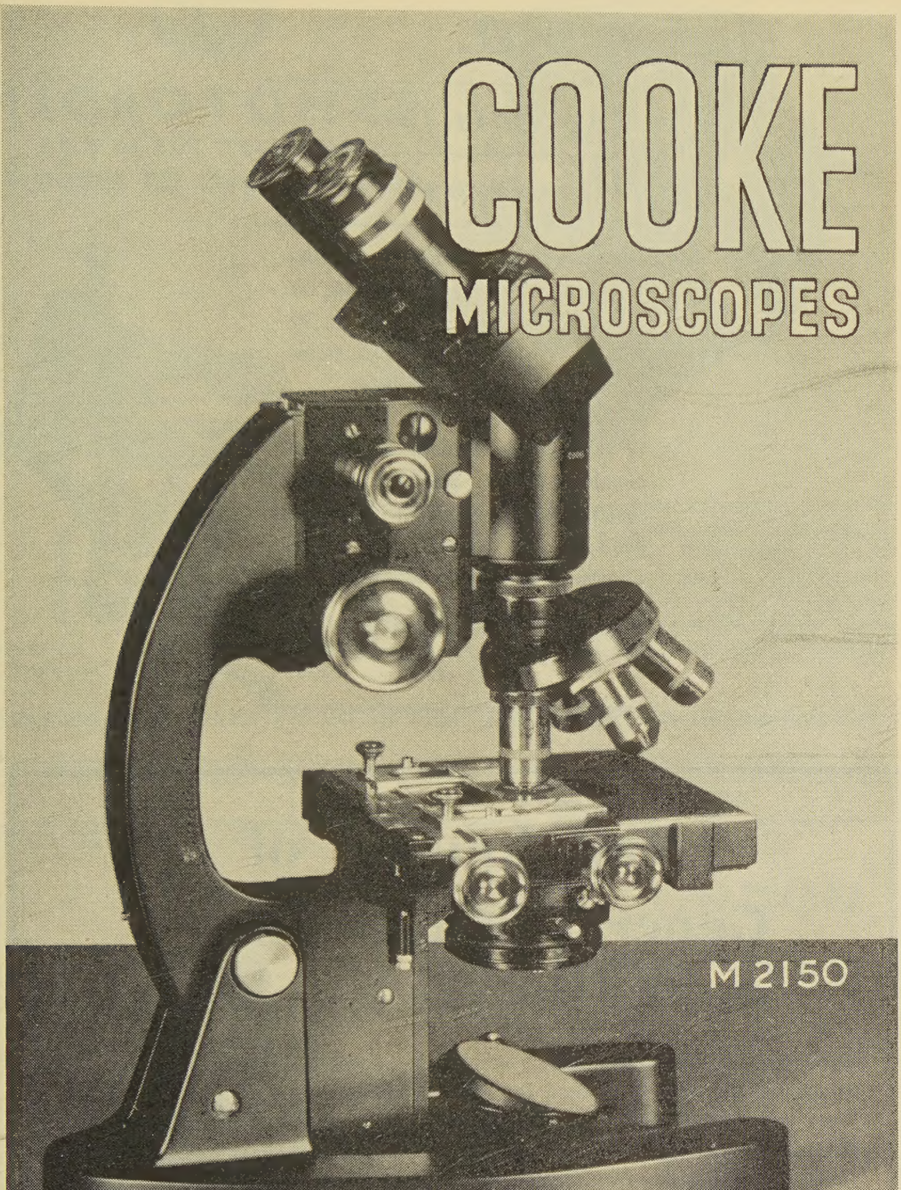
A constantly changing large stock of recent editions of scientific books on show. Libraries or small collections bought.

H. K. LEWIS & Co. Ltd.

136 GOWER STREET, LONDON, W.C. 1

Telephone: EUSton 4282 (7 lines) *Established* 1844

Business hours: 9 a.m. to 5 p.m., Saturdays to 1 p.m.


COOKE
MICROSCOPES
M 2150
Cooke Troughton & Simms
LTD
YORK ENGLAND

B.D.H.

Materials for Microscopy

More than one hundred specially prepared reagents and materials for microscopical work are supplied by B.D.H. — as well as B.D.H. stains and B.D.H. staining solutions.

Notes on recommended methods of staining are included in the free booklet ‘B.D.H. Standard Stains’ together with a priced list of B.D.H. Materials for Microscopical work obtainable, on application, from

THE BRITISH DRUG HOUSES LTD.
B.D.H. LABORATORY CHEMICALS GROUP
POOLE DORSET

St/5

The Journal of General Microbiology

Editors: Dr. B. C. J. G. Knight and Dr. A. A. Miles

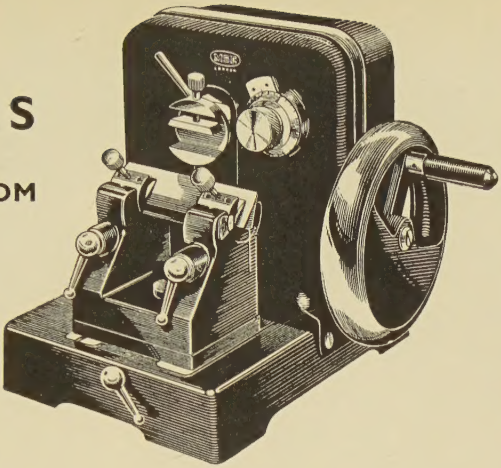
The *Journal of General Microbiology* is conducted by the Society for General Microbiology, whose aim is to promote the advancement of microbiology by providing a common meeting ground for those working in all the various specialized fields including, for example, medical, veterinary, agricultural and economic microbiology. The Society is concerned with the more fundamental aspects of the study of bacteria, viruses, micro-fungi, protozoa and microscopic algae in their various biological activities, its members being united by the common aim of developing scientific knowledge concerning micro-organisms.

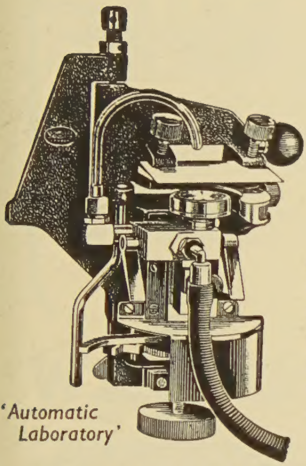
The subscription rate is 50s. net per volume of 3 parts, Single parts 20s. each, plus postage. Orders should be sent to

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W. 1

MSE
LONDON

MICROTOMES
FOR EARLY DELIVERY:
4 MODELS TO CHOOSE FROM

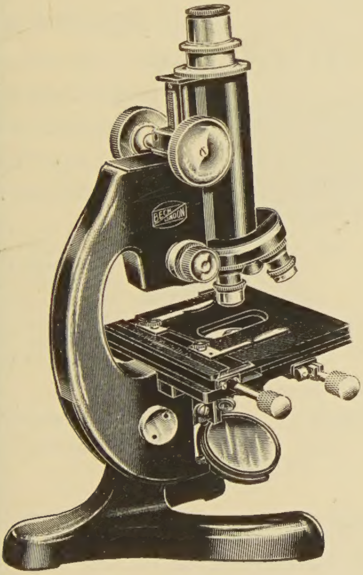




'Automatic Laboratory'

MSE Microtomes include the 'Automatic Laboratory' for paraffin or freezing work, the 'Rotary' (Minot type) for fast accurate serial sectioning, the 'Precision Plano' (Base Sledge type) for paraffin, celloidin or freezing work, and the MSE 'Large Section' Microtome (according to Gough and Wentworth). The last mentioned makes possible a new technique in sectioning—the making of sections as large as 11″ × 9″ for direct observation. Full details of any model on request.

MEASURING & SCIENTIFIC EQUIPMENT Ltd., 14–28 Spenser St., London, S.W.1



BECK

No. 47 LONDON
MICROSCOPE

Pre-eminent
in
Design
Workmanship
and
Performance

Descriptive booklet on application

●

R. & J. BECK LTD.
69 MORTIMER ST., LONDON, W.1

REVECTOR
DYES

are

Standard Products
for use in Biological
Staining and for other
scientific purposes

•

*Each item is
identified by its
Colour Index
Number*

•

*Obtainable from your
usual dealer or from*

HOPKIN & WILLIAMS LTD.

FRESHWATER ROAD
CHADWELL HEATH
ESSEX

The Journal of

Experimental Biology

Edited by J. GRAY

¶ The official medium for the publications of the Society for Experimental Biology, including articles on experimental biology and comparative physiology, whether zoological or botanical.

¶ The Society is affiliated to the Biological Council and the Journal is included in the Council's 'Contents Lists' service.

¶ The subscription rate is £3. 3*s*. net per volume. Single numbers cost £1. 1*s*. net, double numbers £2. 2*s*. net each.

¶ The current issue is Volume 27, No. 2, September 1950.

CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1

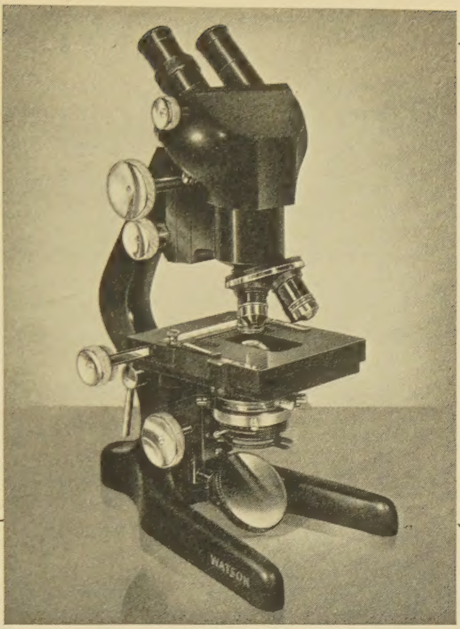
THE
'BACTIL' MODEL

The new binocular microscope of modern design incorporating new features, and still retaining the high standard of precision and rigidity for which these instruments are well known.

For full particulars write for booklet IA which will be sent post free.

WATSON'S

W. WATSON & SONS LTD. 313 HIGH HOLBORN, LONDON, W.C.I.



**Baker's
New Intense
ow-voltage Lamp**

specially designed for
**hotomicrography
ark Ground
nd Phase
icroscopy**

special 6-volt
-amp. bulb is sup-
lied. Large condens-
g lens. Transformer
r resistance for A.C
r D.C. current. Filter
arrier and iris dia-
hragm available.

PRICES:

ith transformer for A.C. . .	£11 12 6
ith resistance for D.C. . .	£11 15 0

Please state voltage

. BAKER of Holborn **Ltd.**
44 HIGH HOLBORN, LONDON, W.C.1
Phone: HOLborn 1427 & 4004

GURR'S
Established 1915

STANDARD STAINS
AND REAGENTS
FOR MICROSCOPY

KNOWN
AND
USED
THROUGHOUT
THE WORLD

Write for Price List Y 16 & Literature

GEORGE T. GURR

Laboratories:

136 NEW KING'S ROAD,
LONDON, S.W. 6

No connexion with any other firm of similar name

JOURNAL OF THE
Marine Biological Association
OF THE UNITED KINGDOM

VOLUME XXIX, No. 2 SEPTEMBER 1950

CONTENTS

N. A. Holme. Population-dispersion in *Tellina tenuis* da Costa.

E. W. Knight-Jones and **J. P. Stevenson.** Gregariousness during settlement in the barnacle *Elminius modestus* Darwin.

H. Barnes and **H. T. Powell.** Some observations on the effect of fibrous glass surfaces upon the settlement of certain sedentary marine organisms.

J. A. C. Nicol. Responses of *Branchiomma vesiculosum* (Montagu) to photic stimulation.

R. Phillips Dales. The reproduction and larval development of *Nereis diversicolor* O. F. Müller.

W. J. Rees. The distribution of *Octopus vulgaris* Lamarck in British waters.

W. A. P. Black. The seasonal variation in the cellulose content of the common Scottish Laminariaceae and Fucaceae.

R. H. Millar. *Lissoclinum argyllense* n.sp., a new ascidian from Scotland.

P. S. B. Digby. The biology of the small planktonic copepods of Plymouth.

Margery Knight and **Mary Parke.** A biological study of *Fucus vesiculosus* L. and *F. serratus* L.

Abstracts of Memoirs. Recording work done at the Plymouth Laboratory.

Marine Biological Association of the United Kingdom. Report of the Council for 1949–50. Balance Sheet. Income and Expenditure Account.

List of Governors, Founders, Members, Honorary and Associate Members.

The Council of the Marine Biological Association wish it to be understood that they do not accept responsibility for statements published in the *Journal* excepting when those statements are contained in an official report of the Council.

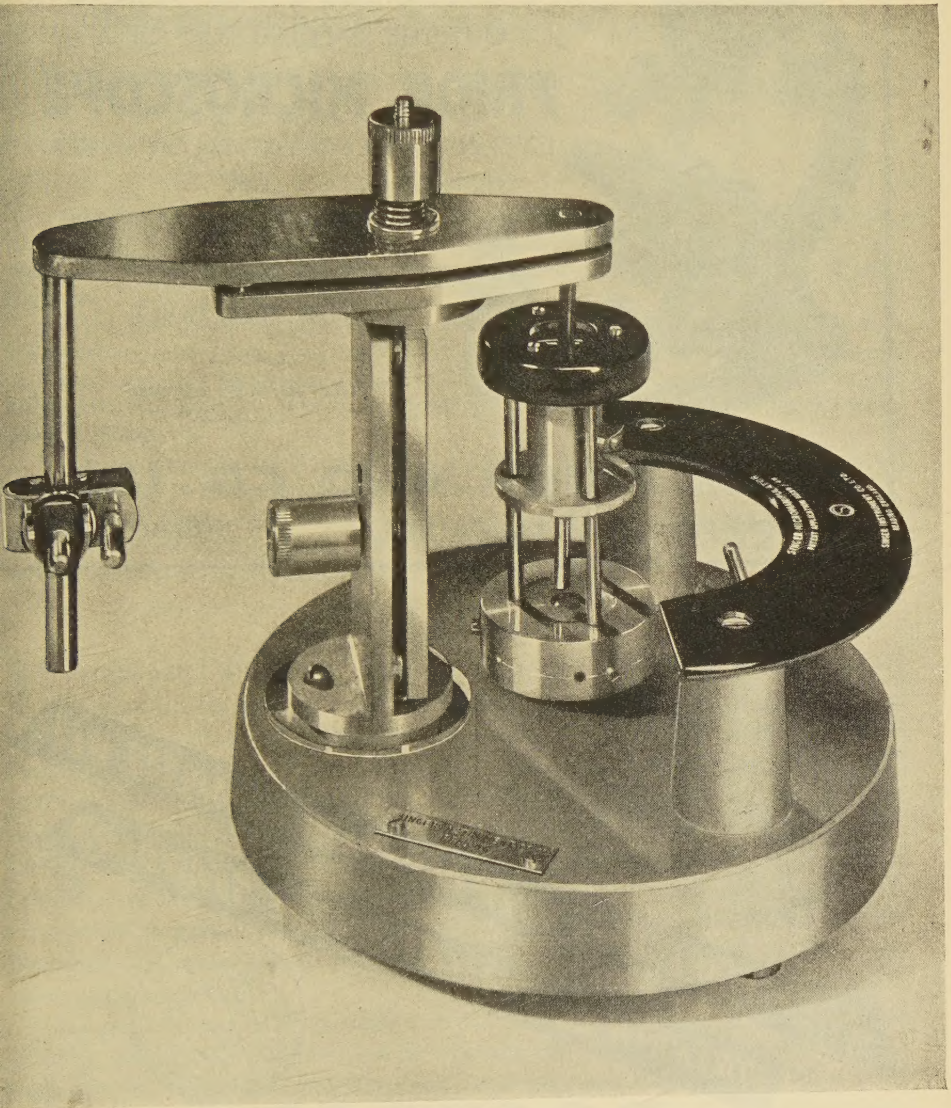
Price Thirty Shillings net post free

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, N.W. 1

SINGER MICROMANIPULATOR

(patent application 11559/48: Foreign patents pending)

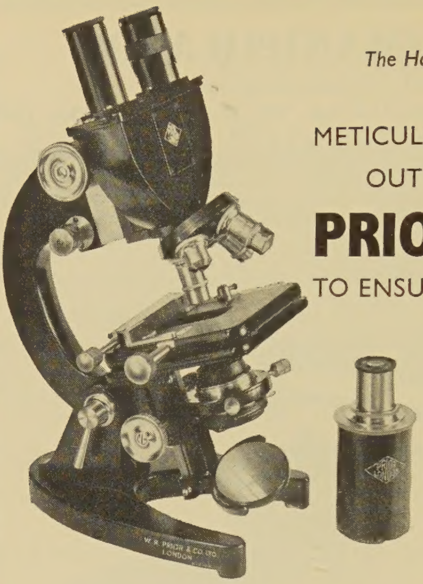
As described by Barer and Saunders-Singer, Q.J.M.S., Vol. 89, pp. 439–47, Dec. 1948



SINGER INSTRUMENT COMPANY LIMITED
FOUNTAIN COURT, 83 LONDON STREET
READING, BERKSHIRE, ENGLAND

Manufacturers also of

Singer Microdissector (patent application 14125/50: Foreign patents pending)



The Hall-mark of a Precision-built Microscope

METICULOUS CARE IS TAKEN THROUGHOUT THE MANUFACTURE OF A

PRIOR MICROSCOPE

TO ENSURE THE PERFECTION ON WHICH THE
PRIOR REPUTATION
FOR QUALITY
HAS BEEN BUILT

Literature on request

W. R. PRIOR AND CO. LTD.

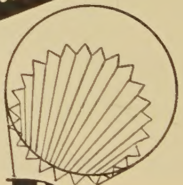
28A Devonshire Street, London, W.1 **WELbeck 4695**

RESIN *Toughened*

A NEW FIBREFREE FILTER PAPER—THE COMPLETE WET-STRENGTH EQUIVALENT OF THE STANDARD

POSTLIP *mill* **633**

EVANS ADLARD & CO. LTD. WINCHCOMBE·GLOS.



Observations on the Ligament of *Mytilus edulis*

By E. R. TRUEMAN

(*From the Department of Zoology, University College, Hull*)

SUMMARY

1. The ligament of *Mytilus edulis* is situated between the valves of the shell immediately below the dorsal margin and may be divided into two main layers, the inner and the outer, the principal features of which appear to correspond with those of other bivalves.

2. The outer layer is subjected to a tensile strain transverse to the longitudinal plane of the shell, which is imposed by the addition of the inner layer. This strain is increased when the valves are closed. At the same time the inner layer is compressed. It is significant, however, that the birefringence of the main outer layer is much smaller than in *Tellina*.

3. The prodissoconch has a small internal ligament which is replaced by the normal adult structure as the posterior dorsal margin of the young mussel is extended.

4. The axis about which the valves open is situated along a line drawn between the two main layers. Anteriorly the adult ligament becomes split and ceases to function. Possible explanations are suggested.

INTRODUCTION

IN recent years little work has been carried out on the ligament of bivalves and that which has been done is largely the work of palaeontologists (e.g. Newell, 1937 and 1942). Among earlier work reference should be made to the important description of some features of the morphology of the ligament of *Mytilus edulis* by Villepoix (1892), to the account of Reis (1902), and to the more recent descriptions of Field (1922), White (1937), and Werner (1939). Other workers such as Jackson (1890), Dall (1889 and 1895), and Bernard (1895–7) discussed the ligament in general terms, the latter with some reference to development. The author has previously published observations on the ligament of *Tellina tenuis*, which is wholly external and parivincular (Trueman, 1942 and 1949).

This paper contains a brief account of the structure of the adult ligament of *Mytilus edulis* in which some new features are described, together with observations on its operation, development, and possible homologies. Results of work on the nature of the horny material, *conchiolin*, of the shell and ligament, will be published later. The term conchiolin is used in this paper in the most general sense to indicate the non-calcareous material of the shell and ligament.

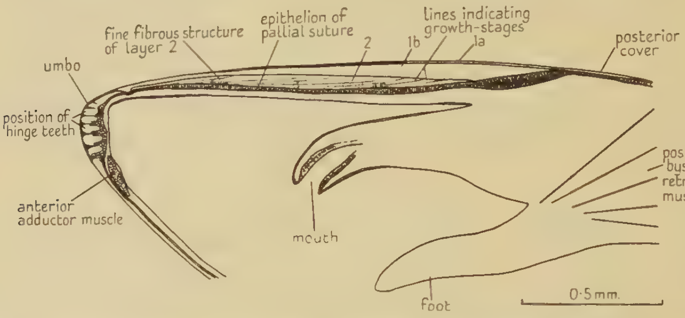
The methods used in this investigation are similar to those previously described (Trueman, 1949). Descriptions and drawings of the ligament have been made from serial sections cut in ester wax and stained in Mallory's triple

[Quarterly Journal of Microscopical Science, Vol. 91, part 3, September 1950.]

stain. The specimens used ranged from 0·25 mm. to 13 mm. in length. Comparison was made with larger mussels which are inconveniently long for serial sections.

GENERAL DESCRIPTION

The ligament, which extends along the dorsal margin of the valves for just less than half of the length of the shell (Text-fig. 1), is situated between the valves and does not project above them. In transverse section it is nearly rectangular (Text-fig. 3), in marked contrast to the shape of a wholly external ligament (e.g. *Tellina*). Like such a ligament, however, it consists of two main layers, comparable with the outer (1) and the inner (2) layers respectively of the *Tellina* type of ligament. In *Mytilus edulis* the outer may be subdivided into layers 1a and 1b. These layers show many features similar to those of the corresponding parts of the ligament of *Tellina tenuis* (Trueman, 1949).

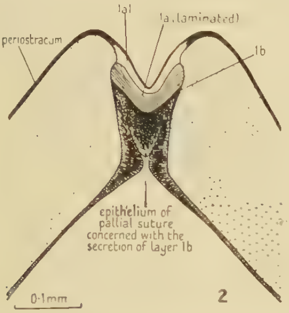

fine fibrous structure of layer 2
epithelion of pallial suture
lines indicating growth-stages
2
1b
1a
posterior cover
umbo
position of hinge teeth
anterior adductor muscle
posterior bussal retractor muscle
mouth
foot
0·5 mm.

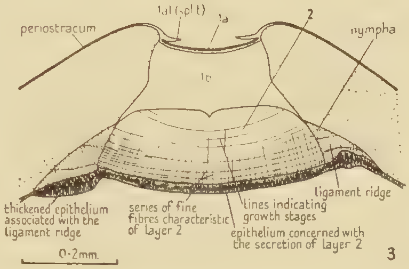
TEXT-FIG. 1. Median longitudinal section through the anterior region of *Mytilus edulis*, showing the position of the hinge ligament in relation to its general anatomy.

Layer 1a is formed at the posterior end of the ligament near the fusion of the mantle lobes and extends forwards first as a 'posterior cover' (Trueman, 1949), then as the dorsal and outermost layer of the ligament (Text-fig. 2). This layer is laminated, the laminae being most clearly seen in the posterior region of the ligament (Text-fig. 2). The outer part of layer 1a is referred to in this paper as layer 1a1 and it is probably equivalent to layer 1a1 of the ligament of *Tellina*. The periostracum of each valve is attached to the lateral margin of this layer (Text-figs. 2 and 3). In the ligament of the mussel but not in that of *Tellina*, layer 1a1 becomes split in the mid-line above the posterior part of layer 2. The remainder of layer 1a is thus exposed along the dorsal surface of the ligament (Text-fig. 3).

Layer 1b forms the main part of the outer layer (1), extending from the umbo to near the posterior margin of the ligament. The shape and general relationships of this layer may be best understood from Text-figs. 1, 2, and 3, 1b. This layer is sharply marked off from the shell laterally and there seems to be little adhesion of the valves to the ligament except by means of layer 1a and the periostracum.

Layer 2 is situated below all but the posterior part of layer 1b (Text-figs. 1 and 3). In sections it has a fine fibrous appearance with the fibres running perpendicular to the ventral surface (Text-figs. 1 and 3). Lines nearly parallel to the latter indicate growth stages. As layer 2 is secreted a calcareous (White, 1937) or ligament ridge is deposited along each valve. These structures tend to prevent the ligament from moving in a ventral direction. They are nacreous and vacuolated (see Villepoix, 1892). The inner layer (2) is attached laterally to the ligament ridge upon a flat surface, termed the nympha (Text-fig. 3).


1a1
1a (laminated)
periostracum
1b
epithelium of pallial suture concerned with the secretion of layer 1b
0·1mm
2


1a1 (split)
1a
2
periostracum
nympha
1b
ligament ridge
thickened epithelium associated with the ligament ridge
series of fine fibres characteristic of layer 2
lines indicating growth stages
epithelium concerned with the secretion of layer 2
0·2mm.
3

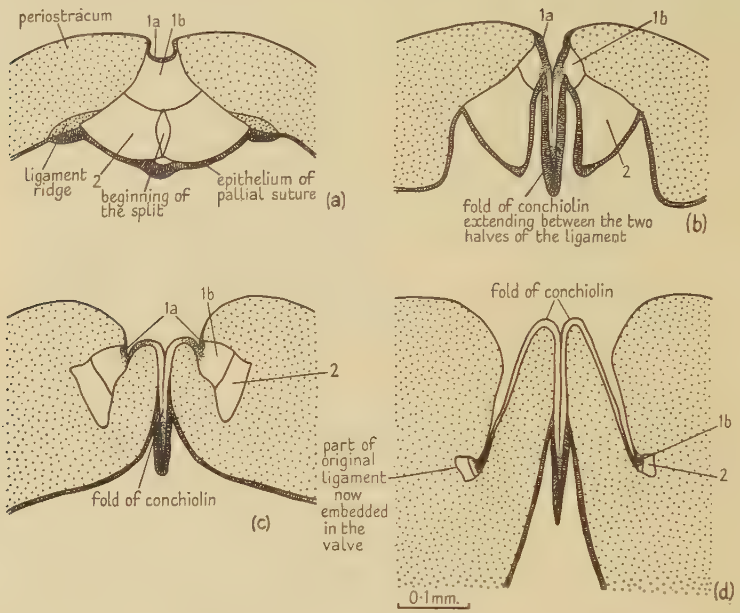
TEXT-FIG. 2. Transverse section of the ligament in the region of the posterior cover showing the periostracum, shell (stippled in this and subsequent diagrams), and the posterior cover which is represented by layers 1a1 and 1a. Deposition of layer 1b is just beginning. Layer 1a1, which is attached laterally to the periostracum, subsequently becomes split. Layer 1a shows characteristic lamination. This figure and the one following are drawn from serial sections of a specimen 13 mm. long, detail of the shell being added from ground sections.

TEXT-FIG. 3. Transverse section of the normal ligament of *Mytilus edulis* showing the various layers and structures mentioned in the text.

With Mallory's triple stain layers 1b and 2 are readily differentiated, the former staining red and the latter blue. This is comparable with the staining reactions in *T. tenuis* previously described (Trueman, 1949) and those of the ligaments of certain other bivalves which have been examined, e.g. species of *Cardium*, *Sphaerium*, *Anodonta*, *Ostrea*, and *Nucula*. The epithelium of the pallial suture concerned with the secretion of the layers of the ligament is similar in *Tellina* and *Mytilus* (Text-figs. 1, 2, and 3).

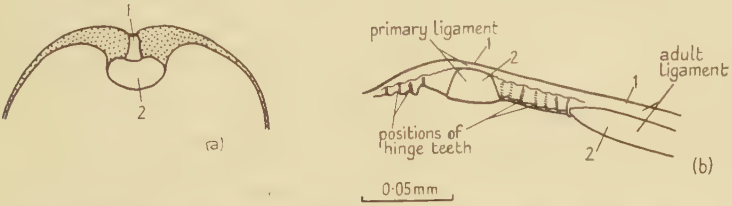
In shells of more than 5 mm. length the anterior end of the ligament becomes progressively divided down the mid-line into two halves. This phenomenon is shown in Text-fig. 4 in a series of transverse sections of a specimen 13 mm. long; a is the most posterior section and d the most anterior. The stages shown in this figure are equivalent to those through which any part of a ligament passes when becoming split. The fissure first occurs in the inner layer (Text-fig. 4a) and extends to the outer layer as the ligament is pulled apart. Into the gap thus formed the epithelium of the pallial suture secretes a fold of conchiolin (Text-fig. 4, b, c, and d) which is similar to the outermost layer (1a). Immediately posterior to the split portion of the ligament the inner layer (2) is secreted. The reasons for this change in the nature of the secretion are of interest. They are probably related to the physical and chemical properties of the layers concerned. Layers similar to layer 1a appear to be characteristic of the outer surfaces of the bivalve. Those similar to layer 2 are typically internal.

After the fissure has developed the calcareous shell is deposited around each half of the ligament which becomes deeply embedded in the valve (Text-fig. 4, c and d). The fold of conchiolin is extended and eventually separates the hinge teeth. Similar features are found in *Modiolus modiolus*. Their possible interpretation is discussed below.


periostracum
1a
1b
ligament ridge
2
beginning of the split
epithelium of pallial suture
(a)
1a
1b
2
fold of conchiolin extending between the two halves of the ligament
(b)
1b
1a
2
fold of conchiolin
(c)
part of original ligament now embedded in the valve
fold of conchiolin
1b
2
0·1mm.
(d)

TEXT-FIG. 4. A series of sections of the anterior end of the ligament of a specimen of *Mytilus* to show stages in the formation of the split in the ligament. a is the most posterior section and shows the fissure beginning in layer 2. b, 0·23 mm. in front of a, shows the split well developed with a fold of conchiolin extending between the two halves. In c and d these have become more separated and the inner calcareous layer of the shell is secreted between them. Section c is 0·1 mm. in front of b, and d 0·1 mm. in front of c.

In larval stages (0·25 mm. long) the ligament is not divisible into the two characteristic main layers (1 and 2). The valves are joined together by the periostracum and by a layer of about 2μ in thickness, situated below the former, which appears to be similar to the outer layer (1b) of the adult shell. The inner layer (2) was first observed in young specimens of length 0·5–1 mm. (i.e. immediately after spatfall). The position of the early ligament has been described by Bernard (1895–7) who made the important observation that it is invariably internal when it appears in the young pelecypod. The primary ligament of the young mussel (Text-fig. 5) is a small internal structure and clearly consists of outer and inner layers, which correspond with those of the adult ligament. It is situated immediately below the umbones, and hinge teeth or *crénelures* (Bernard, 1896) can be observed both in front of and behind this primary ligament, somewhat resembling a form of taxodont dentition. The adult ligament is secreted in a shallow pit situated in the region of the posterior hinge teeth. In mussels 0·5–2 mm. long the outer layer extends between the valves in a similar manner to the posterior cover of *Tellina* (Trueman, 1949). The primary ligament consists of this outer layer, slightly thickened, and a wedge of inner layer (2). The latter is not continuous with the corresponding part of the main ligament (Text-fig. 5b). In the fully grown mussel the primary ligament is surrounded by the shell and is non-functional.



TEXT-FIG. 5. Transverse (a) and longitudinal (b) sections of the primary ligament showing its relationship with the adult ligament and the hinge teeth.

THE GROWTH OF THE LIGAMENT

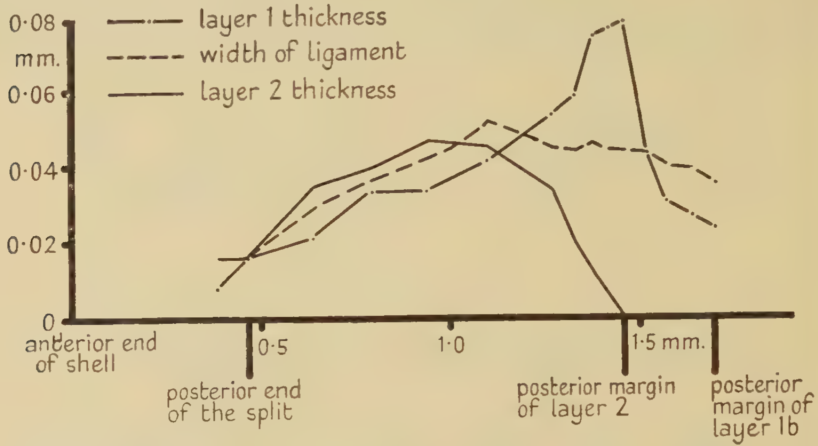
As in the case of *Tellina* deposition of the outer layer extends forwards from its posterior margin to the posterior end of the inner layer. The rapid increase in thickness between these two points is clearly shown in Text-fig. 6. The outer layer is extended posteriorly as the mussel grows, whereas the inner is also increased in thickness along most of its length.

Measurements of the thickness of the outermost layer (1a) show a considerable reduction after deposition of the inner layers (1b and 2). The latter probably cause a tensile strain in layer 1a, which thus behaves similarly to and is homologous with the posterior cover and layer 1a of *Tellina* (Trueman, 1949. See fig. 13). Layer 1b shows little modification after secretion. As described above the outermost layer (1a1) of the ligament of *Mytilus* becomes split in the mid-line above the posterior margin of the inner layer (2) (Text-figs. 2 and 3). This is probably due to the increase of tensile strain in the transverse plane of the ligament as the inner layers (1b and 2) are secreted. The splitting of layer 1a1 does not occur in the ligament of *Tellina*.

The distance across the dorsal surface of the ligament (Text-fig. 7, AB) or the width of the ligament has been measured in different specimens at various distances from the umbones. The maximum occurs not at the posterior end of the ligament (where layer 1a is thickest) but more anteriorly (Text-fig. 6), the distance thus being increased during deposition of the inner layer. The dorsal margin of the ligament appears to be stretched in a direction normal to the median plane of the shell.

As the outermost layer 1a is stretched it becomes anisotropic in a similar way to that observed in *Tellina*. Layer 1b is anisotropic only when the inner layer is secreted on its ventral surface. The birefringence of layer 1b in *Tellina* is considerably greater than that of the same layer of *Mytilus*, the maximum value determined in transverse section being 0·005, as compared with 0·022 in *Tellina* (Trueman, 1949).

It may be concluded that post-depositional stretching of the outer layers (1a and 1b) takes place in the ligament of *M. edulis*. It occurs more markedly in the external parivincular type of ligament, e.g. *Cardium* or *Tellina*. The higher value of birefringence in the latter is possibly associated with the greater amount of stretching of their outer layers.



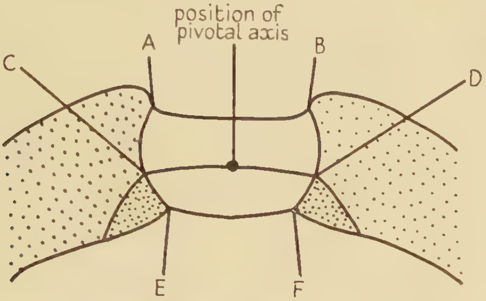
TEXT-FIG. 6. Graph showing the relationship of the thickness of the main layers (1 and 2) and of the width of the upper surface (Text-fig. 7, AB) of the ligament to each other and to the other features indicated.

SOME OBSERVATIONS ON THE OPERATION OF THE LIGAMENT

Observations were made of the operation of the ligament by use of transverse sections of whole specimens in which all muscle attachments had been cut so that the opening of the valves could be controlled. The outer layer (Text-fig. 7, AB) was measured in both the closed and the normal feeding position of the valves. When the valves are closed from the latter position the upper surface of the ligament is visibly stretched in the transverse plane, the width being increased by about one-eighth (measured on a section of a ligament 25 mm. behind the umbo of a mussel 70 mm. long). Simultaneously the width of cross-section of the ventral edge of the inner layer (Text-fig. 7, EF) is reduced by approximately the same amount. Where layers 1 and 2 are of equal thickness the transverse measurement along their common boundary (Text-fig. 7, CD) remains constant when the valves are opened and closed. From these and other observations it is probable that the pivotal axis of the ligament, about which the valves open, lies between layers 1 and 2 in the mid-line (Text-fig. 7), as in *T. tenuis*.

About two-thirds of the ligament of an adult mussel is effective in causing the opening of the valves. There are two non-functional regions, one at each end of the ligament. Anteriorly the split portion together with the ligament immediately behind it no longer operates the valves; posteriorly layer 1 does not open the shell prior to deposition of the inner layer (2). From such observations as have been made the presence of both the outer and the inner layers seems to be necessary in ligaments of most bivalves.

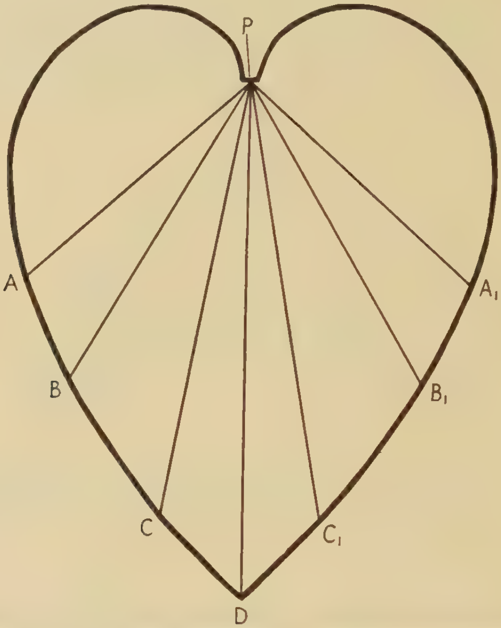
Dall (1895) pointed out that all hinge ligaments must be arranged along a straight axis, as no bivalve ligament can function around a curve. Thus most external hinge ligaments are situated along the straight dorsal margin of the shell, and many bivalves without this characteristic feature possess an internal ligament.



TEXT-FIG. 7. Diagrammatic transverse section of the ligament showing the position of the pivotal axis and of various points referred to in the text.

The location of the pivotal axis in the mid-line between the two main layers (1B and 2) (Text-fig. 7) accounts for certain of their characteristics. The outer layer is subjected to tensile stresses when the valves are closed; at the same time the inner layer undergoes compression. The latter is weak to tensile stresses, splitting easily parallel to the fibres (Text-fig. 3). But since this layer (2) is situated below the pivotal axis, it is normally only subjected to compression when the valves are closed.

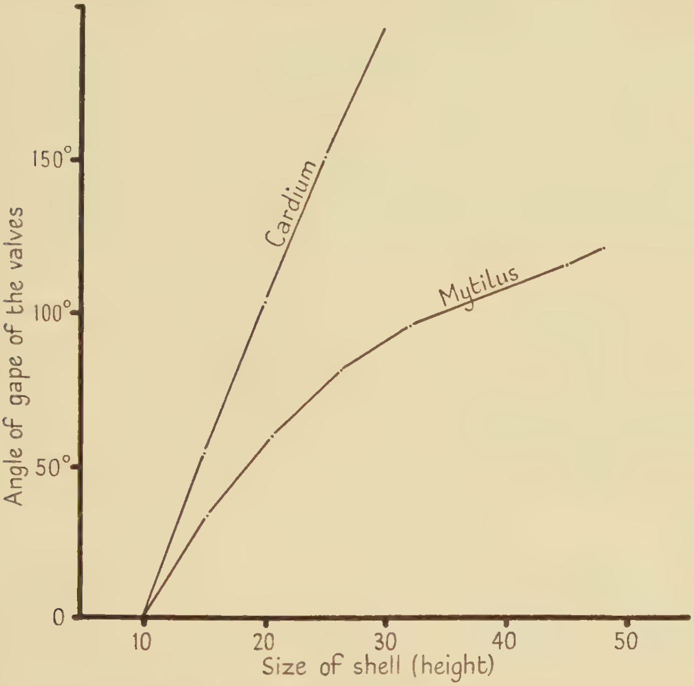
In any consideration of the development of the ligament it is essential to take note of the progressive increase of the angle (Text-fig. 8, APA_1) between the early parts of the valves, which is caused by the secretion of the shell along the ventral margin (Text-fig. 8, AD or A_1D). An attempt has been made to measure the rate of increase of this angle in various genera. A transverse section was cut through the shell in the umbonal region and projected so that an enlarged outline of the valves could be drawn. Measurements were then made of the height of the shell (Text-fig. 8, PA, PB, PC, and PD) and of the corresponding increase of angle of gape at the pivotal axis, represented in Text-fig. 8 by angles $APB+A_1PB_1$, $APC+A_1PC_1$, and APA_1. The amount of angular separation undergone by the valves of a shell in growing from a height of 10 mm. to various subsequent stages was thus determined. Owing to the obliquity of the shell of *M. edulis* measurements were made on sections cut perpendicular to the ligament at the distance from the umbones equivalent to the ligament length of a young mussel of 10 mm. height. With more equilateral bivalves such as *Cardium* the heights can be measured directly from the projected section, but with *Mytilus* it is more convenient to convert this dimension into the true height of the shell.



TEXT-FIG. 8. Diagrammatic transverse section of a bivalve showing the effect of the secretion of the shell at the ventral margin. In the young bivalve points A and A_1 were together and have been separated by the deposition of AD (and A_1D), so that the valves now have a permanent gape of the angle APA_1. Some intermediate stages have been indicated.

The cross-section of a valve of most lamellibranchs corresponds to an equiangular spiral, though a reduction of spiral angle in the later stages of growth is not uncommon. *Cardium edule* and *Barbatia lactea*, for example, have a comparatively large spiral angle, and deposition at the margin of the valves causes a large increase of the angle between the early valves. Those shells with a lower spiral angle, such as *Chlamys opercularis*, have relatively a much lower rate of increase of angle at their pivotal axis. When the stages in the increase in the angle of gape of some young part of a shell (e.g. representing the stage of 10 mm. height) are plotted against the corresponding heights of the shell, e.g. with *Cardium*, the curve produced is similar to that obtained from comparable measurements made on an equiangular spiral (Thompson, 1942, p. 749). The curve resulting from these measurements in *Cardium edule* corresponds approximately with one based on a logarithmic spiral of 55° spiral angle (Text-fig. 9).

The corresponding curve based on similar measurements in *Mytilus edulis* is more complex, showing a marked change in the rate of increase in the gape of an early stage in the shell. This results from the fact that the section of the young shell of *Mytilus* approximates to a spiral angle of about 35° which is reduced relatively suddenly in large shells (Text-fig. 9). This implies that, in adult stages, the rate of increase of the gape of an early stage of *Mytilus* is markedly slower than in many lamellibranchs.



TEXT-FIG. 9. Graph of the height of the shell, in mm., plotted against the increase of angle of gape (in degrees) at point P (Text-fig. 8) for two bivalves *Mytilus edulis* and *Cardium edule*. For full description see the text.

DISCUSSION

It may be observed that the ligament of *Mytilus* has a very significant split even though the rate of opening of the valves, especially during later growth, is relatively low. The whole of the original ligament of a shell 16 mm. long becomes split when the bivalve has grown to a length of 70 mm. While this growth is taking place the valves have increased their angle of gape by 120°. The ligament of a specimen of *Cardium edule*, 12 mm. long, becomes entirely split when the valves have opened during growth to an angle of about 200°. Such a split only affects the inner layer (2) of the ligament. It appears that the progressively increasing gape of a growing shell affects differently the different types of ligaments.

The dorsal surface of the ligament of *M. edulis* is slightly arched at the anterior end when viewed in the plane of a sagittal section. As the gape of the early valves increases the umbones move away from each other. The anterior part of the ligament is completely below the pivotal axis (owing to the slight arching) and upon the opening of the shell both layers (1 and 2) of the ligament tend to be pulled apart. If the ligament were not arched at the anterior end, then the opening of the valves, during growth of the shell, would be about the normal pivotal axis of the ligament. Layer 2 would thus be subjected to an increasing tensile strain as the valves open and layer 1 would probably be only little affected. The latter seems likely to be the conditions found in *C. edule*. As observed above, the ligament of the mussel becomes deeply embedded in each valve after it has split. This is a process which progresses as the shell grows and gives rise to the much thickened sub-umbonal region of the shell.

It may be deduced that the ligament of the mussel splits because of its slight dorsal arching coupled with the increasing gape of the valves. The comparatively long ligament of *M. edulis* must be related to the development of the mytiliform shell with a long posterior end. The length of the ligament may to some extent be accounted for by its internal situation. Because of this it is probably less efficient than the external parivincular type of ligament of *Tellina* or *Cardium*.

The ligament of an adult mussel begins to develop at about the time of spatfall, as the posterior dorsal border of the shell extends. The internal, primary ligament appears to function only when the shell is small and circular. If the shell had remained more or less equilateral in shape it might possibly have persisted as an 'internal' type of ligament situated in the umbonal region only, such as, for example, that of *Ostrea*. A full discussion of the problem of the relationship of ligament form and efficiency to the shell shape and to the influence of the environment will be published later.

ACKNOWLEDGEMENTS

The writer is indebted to Professors P. G.'Espinasse, and C. M. Yonge, F.R.S., for criticism of the manuscript and to Dr. A. F. Hallimond for help in crystallographic determination. The material was collected at the Marine Station, Millport, and the author is grateful for the assistance of the staff. Mr. C. B. Rees kindly supplied specimens of the spat of *Mytilus* which he had identified.

REFERENCES

BERNARD, F., 1895. Bull. Soc. géol. Fr., **23,** 104.
—— 1896. Ibid., **24,** 54 and 412.
—— 1897. Ibid., **25,** 559.
DALL, W. H., 1889. Amer. J. Sci., **38,** 445.
—— 1895. Trans. Wagner Inst. Sci. Philad., **3,** 486.
FIELD, I. A., 1922. Bull. U.S. Bur. Fish., **38,** 127.
JACKSON, R. T., 1890. Mem. Boston Soc. nat. Hist., **4,** 277.
NEWELL, N. D., 1937. Univ. Kansas Publ., **10,** 1.
—— 1942. Ibid., **10,** pt. 2, 1.
REIS, O., 1902. Jh. Ver. vaterl. Naturk. Württemb., **58,** 179.
THOMPSON, D'A. W., 1942. *On Growth and Form.* Cambridge (University Press).
TRUEMAN, E. R., 1942. J. R. micr. Soc., **62,** 69.
—— 1949. Proc. zool. Soc. Lond., **119,** 719.
VILLEPOIX, R. M. DE, 1892. J. Anat., Paris, **28,** 461 and 582.
WERNER, B., 1939. Zool. Jb. Abt. Anat., **66,** 1.
WHITE, K. M., 1937. L.M.B.C. Mem., **31,** 20.

The Structure of the Foetal Yolk-sac Splanchnopleur of the Rabbit

By B. MORRIS

(*From the Department of Zoology, University College of North Wales, Bangor*)

SUMMARY

1. At the 16th day *post coitum*, a remnant of the bilaminar omphalopleur is present in the form of a short tag. The further shortening of this tag is effected by two concurrent processes; firstly the degeneration at its free end, and secondly the pulling out of the ectodermal and endodermal epithelia of which it is composed. This latter process is accompanied by the further growth of mesoderm in the vicinity of the tag, and leads to the appearance of squamous cells in the endodermal epithelium of the yolk-sac splanchnopleur.

2. The active production of multinucleate spherules by the chorionic ectoderm is decreasing in intensity by the 24th day. At this stage the vitello-allantoidean vessels in the mesoderm of the chorion, linking the omphaloidean and placental circulations, have mostly disappeared, only a few small vessels remaining.

3. The apical concentration of the eosinophilic granules and mitochondria in the cytoplasm of the endodermal cells of the yolk-sac splanchnopleur, and the frequent basal position of the nucleus, suggests a secretory polarity. The manner in which the endodermal epithelium is folded and pleated suggests an absorptive function.

4. On the side next to the exocoel the yolk-sac splanchnopleur is lined by a coelomic mesothelium of flattened cells which form a continuous pavement epithelium.

5. The entry of circulating maternal antibodies into the foetal circulation is discussed.

INTRODUCTION

IT has been shown that the passage of circulating maternal antibodies into the foetal circulation during pregnancy in the rabbit takes place exclusively by way of the uterine lumen and the yolk-sac splanchnopleur (Brambell, Hemmings, Henderson, Parry, and Rowlands, 1949). When immune sera are injected into the uterine lumen of pregnant rabbits at 24 days, the foetal yolk-sac splanchnopleur shows a high degree of selectivity of the contained antibodies according to the species in which they were produced (Brambell, Hemmings, Henderson, and Rowlands (in press)). Thus, rabbit antibodies are freely admitted to the foetal circulation, whereas bovine and equine antibodies are almost completely, but not entirely, excluded. At earlier stages this selectivity of the splanchnopleur is less marked.

A reinvestigation of the structure of the foetal yolk-sac splanchnopleur of the rabbit is obviously desirable in view of these new discoveries concerning its function. Morphologically it is certainly not a simple homogeneous membrane, but is a highly complex structure histologically, including endodermal and mesodermal components. Despite the large amount of attention that has

[Quarterly Journal of Microscopical Science, Vol. 91, part 3, September 1950.]

been given in the literature to the embryology of the rabbit it is curious that since the pioneer work of Duval (1889–92) and the work of Branca (1923), little attention has been paid to the structure of the yolk-sac splanchnopleur. Such references as occur are mainly in papers primarily devoted to other problems, such as the well-known work of Sansom (1927) on the giant cells.

It is the purpose of this paper to give a fuller account of the morphological relations and histological structure of this membrane during the later stages of pregnancy in rabbits, with special reference to the physiological findings previously mentioned.

TECHNIQUE

Serial sections of 16-day and 24-day rabbit embryos, *in situ* in the uterus and membranes, were prepared. The material was fixed in aqueous Bouin's fluid and stained in Ehrlich's haematoxylin and aqueous eosin. Isolated membranes were collected from experimental animals killed at 24 days. These animals had been killed by an intravenous injection of magnesium chloride into the ear vein. Membranes were either transferred immediately to fixative or were first washed in isotonic saline. Whole mounts were made of some specimens impregnated with silver nitrate. These membranes were first stretched over glass, rinsed in distilled water, and immersed in silver nitrate, brightly illuminated, for 5–15 minutes. After dehydration and clearing the preparations were mounted in damar. They were examined in surface view.

The remainder of the material was fixed in aqueous Bouin or in Flemming's fluid without acetic acid, embedded in paraffin wax and sectioned at 3–10μ. The Bouin-fixed material was stained in Ehrlich's haematoxylin and 1 per cent. aqueous eosin. The Flemming's without acetic acid fixed material was either stained by Kull's triple stain or post osmicated for 1–8 days, sectioned, cleared, and mounted unstained.

THE ARRANGEMENT OF THE EMBRYONIC MEMBRANES AT THE 16TH DAY

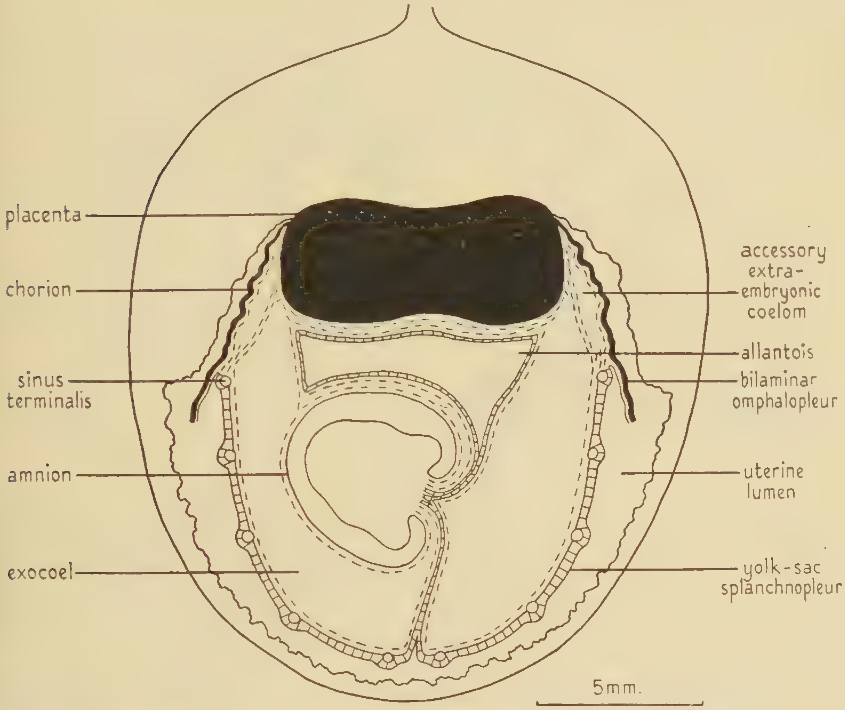
The morphological relations of the embryonic membranes at 16 days *post coitum* are shown in Text-fig. 1. At this stage the allantoic placenta is fully established. The yolk-sac splanchnopleur is completely inverted, and the bilaminar omphalopleur which formed the antimesometrial hemisphere of the yolk-sac wall has disintegrated. A remnant of the bilaminar omphalopleur is still present in the form of a short tag ('la zone residuelle' of Duval), which is attached to the yolk-sac splanchnopleur at a point near to the sinus terminalis.

The chorion extends from close to the sinus terminalis to where it is attached around the base of the placenta. It is lined by allantoic mesoderm, which has split to form inner and outer portions, resulting in the formation of an accessory extra-embryonic coelom. This is 'la zone inter-ombilico-placentaire' of Duval.

The chorion and bilaminar tag, as well as the yolk-sac splanchnopleur, are exposed to the uterine lumen, and hence present possible absorptive surfaces for substances contained therein. Their structures can best be dealt with in separate sections.

THE BILAMINAR OMPHALOPLEUR

At implantation the trophoblast of the antimesometrial hemisphere of the blastocyst adheres to the uterine epithelium at a number of scattered points, penetrates the epithelium and invades the uterine mucosa. Later, at 9–9½ days, the uterine epithelium is sloughed off and degenerates and, simultaneously, a new uterine epithelium is regenerated beneath it, from the epithelial elements in the bases of the uterine glands. This degeneration of uterine tissue takes place at the same time as that of the bilaminar omphalopleur which is closely applied to it. The endoderm of the bilaminar omphalopleur persists for some time after the disappearance of the trophoblast, forming the antimesometrial wall of the yolk-sac (Sansom, 1927).

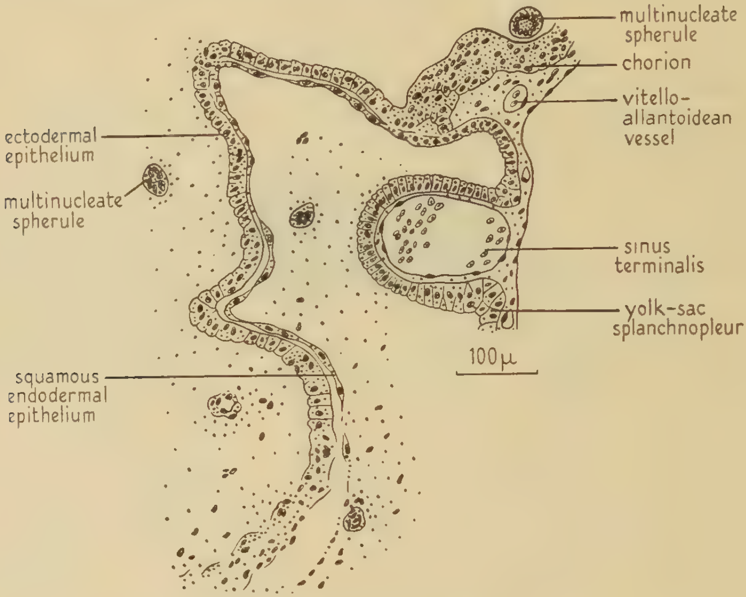


TEXT-FIG. 1. Diagram of a transverse section of the uterus at 16 days, showing the morphological relations of the embryonic membranes.

It would seem reasonable to compare the uterine tissues, destroyed in this way, to the decidua capsularis of other species, since it intervenes between the embryonic membranes and the definitive uterine lumen formed beneath it, which is lined by the regenerated uterine epithelium.

At the 16th day only a small portion of the bilaminar omphalopleur remains (Text-fig. 2). A low columnar epithelium of endoderm cells overlies the sinus terminalis, and is continuous with the squamous endodermal cells of the bilaminar tag. The transition from the low columnar to the squamous form is effected over a comparatively small region, generally not extending for more than twenty cells.

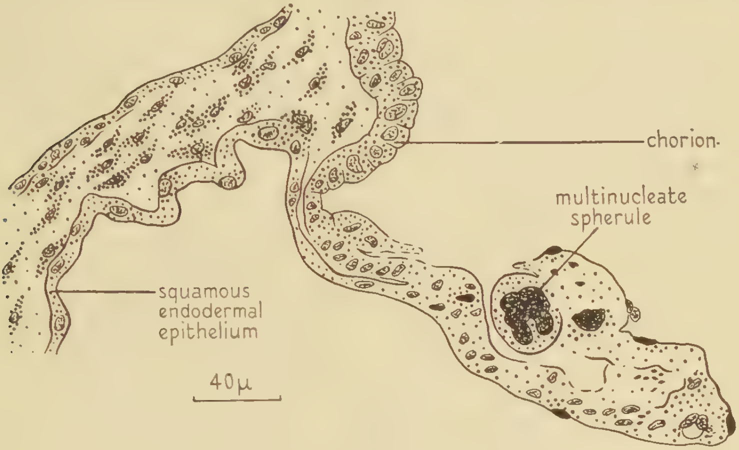
The trophoblastic ectoderm of the bilaminar tag consists of a single layer of cuboidal cells, with large, rounded, centrally placed nuclei. It is continuous with the ectoderm of the chorion. At the end of the bilaminar omphalopleur adjacent to the sinus terminalis the ectoderm tends to become multi-layered, assuming a similar condition to that which exists in the ectoderm of the chorion—to be described later.



TEXT-FIG. 2. The bilaminar omphalopleur at 16 days.

Towards the free end of the bilaminar tag there is considerable degeneration of cell elements in both the ectodermal and endodermal layers. Pycnotic nuclei are present, and the epithelia constituting the tag are being disrupted. The products of this degeneration are being cast off into the uterine lumen. The epithelia near the free end of the tag are frequently not closely applied to one another. As they degenerate they become separated. Near the sinus terminalis a thin wedge of mesodermal tissue is beginning to penetrate between these epithelia.

At the 24th day the bilaminar tag is much shorter, with a considerable accumulation of debris near its free end (Text-fig. 3). Squamous cells, similar to those of the tag, are now present in the endodermal epithelium of the yolk-sac splanchnopleur, and the mesenchyme, which lies beneath them, is more compact than that found elsewhere in the splanchnopleur. The cells of the ectodermal layer are cuboidal, with granular cytoplasm. The accumulation of debris at the free end of the tag is composed of ectodermal cells which are degenerating; the cell outlines of some are still discernible. The debris appears to be loosely attached to the tag by a coagulum, for it retains its position even when the membrane is dissected out of the conceptus. Multinucleate chorionic spherules, in various stages of degeneration, are present in this mass and contribute to its formation.



TEXT-FIG. 3. The bilaminar tag at 24 days.

Duval describes the bilaminar omphalopleur at the end of gestation as a short papilla; the ectodermal and endodermal epithelia, composed of cuboidal cells, being continuous with one another around the free end of the tag. The degeneration at its free end has proceeded up to the limit of the penetration of the mesodermal wedge of tissue, which was seen to be developing at the 16th day.

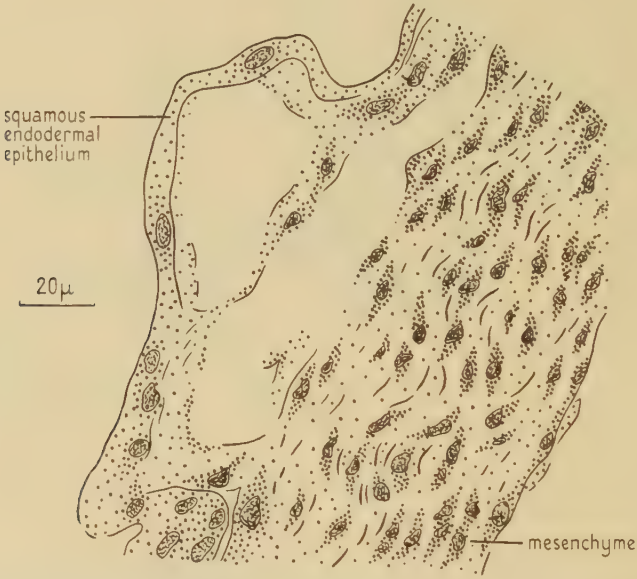
THE CHORION

At $7\frac{1}{2}$–8 days the trophoblastic ectoderm of the mesometrial hemisphere is in contact with the periplacental uterine folds. Laterally and antimesometrially the trophoblast is penetrating the maternal tissue. Between these two areas there is a region of the trophoblast which is not involved in the formation of the placenta, nor does it adhere to the uterine wall. This portion constitutes the chorion læve. At the 10th day it is composed of two layers of cells, outer trophoblastic ectoderm, which is comparatively quiescent up to the 14th day (Sansom, 1927), and an inner layer of mesoderm.

Sansom has described, and we confirm, the active production of multinucleate spherules by the chorionic ectoderm which, at the 16th day, is multilayered. These spherules are cast off into the uterine lumen, where they fragment and degenerate. They are still being formed at the 24th day, but to a lesser extent, and the ectoderm, which is composed of cuboidal cells, is returning to a single-layered condition.

At about the 13th day the chorion is being reinforced by the downgrowth of allantoic mesoderm from the placental region. It reaches the limit of its downward extension at the level of the sinus terminalis by the 14th day (Duval).

The allantoic mesoderm thickens, and by the 16th day has split into two parts, an inner thick vascular portion, and an outer thin layer underlying the chorionic ectoderm. This split results in the production of a so-called accessory extra-embryonic coelom. Blood-vessels are present in the inner layer of mesoderm, and Duval has shown that they anastomose with the sinus terminalis. At the placental end of the membrane they join vessels which penetrate the placenta. Thus there is established, in this region, a connexion between the vitelline and placental circulations.

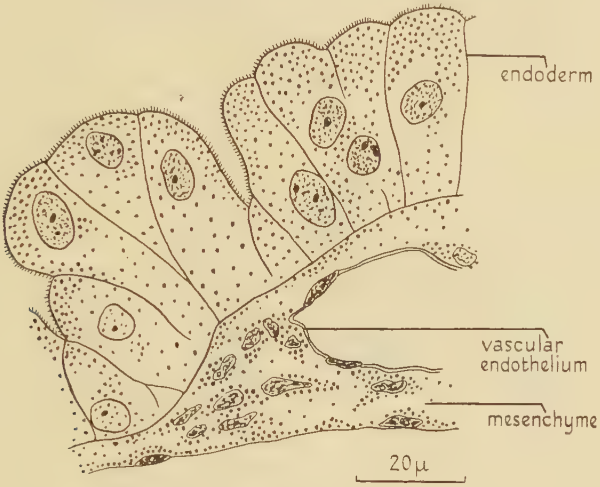


TEXT-FIG. 4. A portion of the yolk-sac splanchnopleur adjacent to the bilaminar tag at 24 days, showing the wavy contour of the cells of the mesenchyme layer.

The inner mesodermal layer thickens progressively and at the 24th day is a more compact tissue than the mesenchyme of the yolk-sac splanchnopleur. It is composed of spindle-shaped cells which are arranged parallel to the surface of the membrane. This layer usually attains its greatest thickness in the region between the sinus terminalis and the base of the bilaminar tag, underlying the squamous and cuboidal cells now present in the endodermal epithelium of the yolk-sac splanchnopleur. Here the linear arrangement of the mesenchyme cells is sometimes interrupted and they assume a wavy contour in section (Text-fig. 4).

At the 16th day the inner mesodermal layer is highly vascular. However, no vessels were observed in this region in sections of 24-day stages. The examination, *in situ*, of the entire extent of this membrane reveals the presence of one or two isolated vessels. It was found (Brambell *et al.*, 1949) that the ligation of the vessels in the yolk-sac stalk did not always completely interrupt the circulation of the area vasculosa, and it was concluded that the circulation was probably maintained by these vitello-allantoidean vessels. The possibility of anastomoses forming between the vessels in the vicinity of the ligature was not excluded.

The compactness of the mesoderm of the chorion at this stage is due, in part, to it being mainly a non-vascular layer. The inner thin mesodermal layer has almost disappeared, but remnants are still visible in some sections.



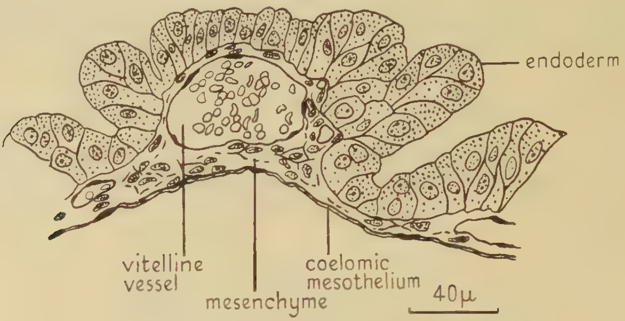
TEXT-FIG. 5. The yolk-sac splanchnopleur at 24 days, showing the brush border of the endodermal cells.

THE YOLK-SAC SPLANCHNOPLEUR

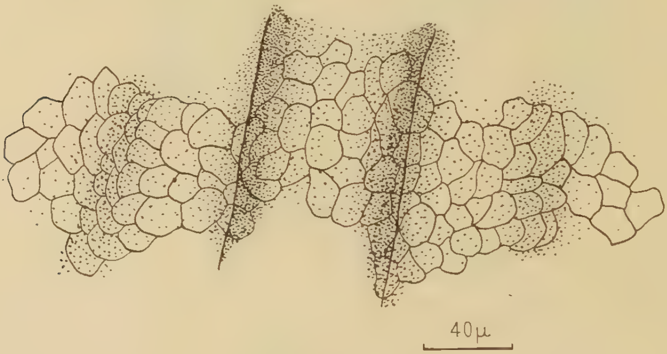
1. *The Endoderm*

At the 16th day the endodermal epithelium of the yolk-sac splanchnopleur is composed of low columnar cells. By the 24th day the epithelium has thickened, the cells having increased in height and diameter, while maintaining the same proportions, being approximately two to three times as tall as they are broad (Text-fig. 5). They form a compact regular layer, without visible intercellular spaces. The flat basal ends of these cells rest upon a distinct basement membrane which separates the endoderm from the underlying mesenchyme. Their convex apical ends are surmounted by a brush border of short, closely set hairs. The larger vitelline vessels, with their covering of endoderm and mesenchyme, protrude into the uterine lumen (Text-fig. 6). In surface view they form prominent ridges (Text-fig. 7). They are present in 16-day stages, and by increasing the surface area exposed to the uterine lumen may serve to facilitate absorption from its contents.

The granules present in the cytoplasm of the endodermal cells increase in size and number up to the 17th day. Their occurrence and distribution in the endoderm of 8- to 22-day stages has been described by Branca (1923). After the 17th day they become fewer and also diminish in size. Branca considers them to be the products of absorption.



Text-fig. 6. The yolk-sac splanchnopleur at 24 days, showing a large vitelline vessel.



Text-fig. 7. The endodermal epithelium of the yolk-sac splanchnopleur at 24 days, in surface view, showing the outlines of an underlying vitelline vessel.

At 24 days they stain readily with eosin. These eosinophilic granules occur throughout the cytoplasm, but are at their greatest concentration distally. A similar concentration of the cytoplasmic fats is observed in the osmicated material. These appear as fairly large black granules of irregular shape throughout the cytoplasm. The membranes stained in acid fuchsin, toluidine blue, and aurantia show that the mitochondria, which are stained dark crimson, are also concentrated apically. The large rounded or oval nuclei are often situated centrally, but are most commonly found nearer to the basal ends of the cells, and they usually contain one or two basic nucleoli. They are considerably larger than the nuclei of the mesenchyme cells.

In surface view the endoderm cells are of irregular shape (Text-fig. 7), and fit closely together to form a continuous layer uninterrupted by intercellular spaces. The amount of intercellular substance present is small.

2. *The Mesenchyme*

The mesenchyme in the region between the sinus terminalis and the bilaminar tag forms a fairly compact tissue, as already described. It is composed of spindle-shaped cells arranged parallel to the surface of the membrane. The boundaries of the cells, constituting the remainder of the mesenchyme, are not easily distinguished in the healthy membrane. In certain membranes, which were damaged by the experimental procedures and were disintegrating, the cells of this layer had become separated and their shapes were more readily discernible. Most commonly the cytoplasm is pulled out into two fairly fine processes, giving a typical spindle shape, but stellate forms are also present. In the healthy membrane tissue spaces occur in the matrix between the cells. The nuclei are of irregular shape.

The amount of cytoplasmic fat present in this layer is small, contrasting with the rather dense concentration in the endoderm. There appears to be little or no differentiation of cell elements.

3. *The Vascular Endothelia*

The vitelline vessels are lined by a vascular endothelium. In sectional view the nuclei of these endothelial cells appear elliptical, staining more intensely with Ehrlich's haematoxylin than the endodermal nuclei. Their cytoplasm is much restricted, even in the immediate vicinity of the nucleus. Together these cells form an exceedingly thin inner lining to the vessels.

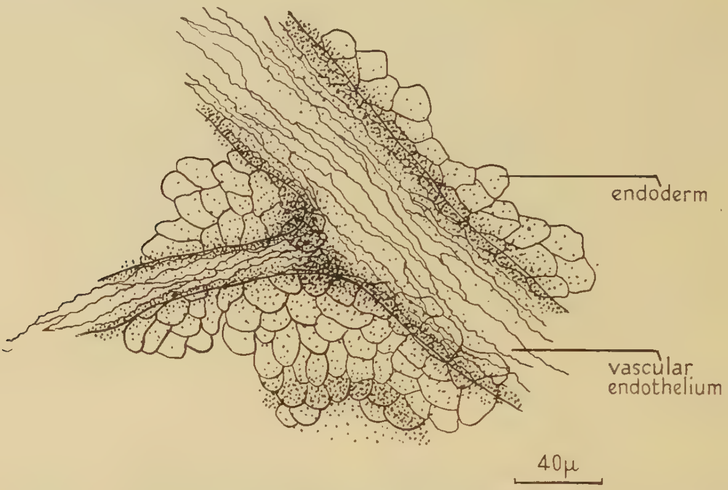
At the 16th day these vessels are full of nucleated embryonic blood corpuscles, but the nucleated condition has been lost by the 24th day. The vessels in the mesoderm of the chorion at the 16th day are of essentially similar structure.

The walls of the endothelial cells are visible in surface view in the silver nitrate preparations. They are elongated cells, often spindle shaped with denticulate outlines (Text-fig. 8). No intercellular spaces were observed, and the amount of intercellular substance present is even less than that in the endoderm.

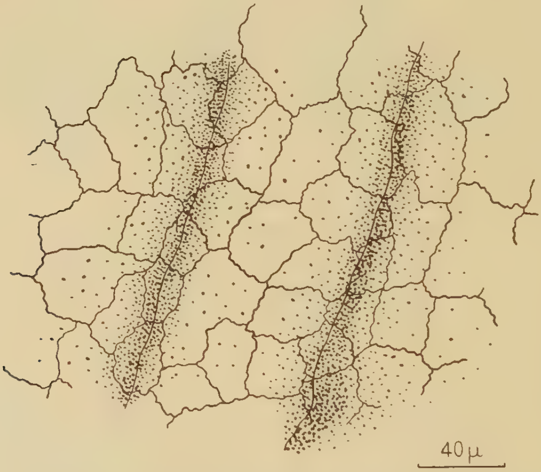
4. *The Coelomic Mesothelia*

A layer of mesothelial cells forms the lining of the yolk-sac splanchnopleur on the side next to the exocoel. In sectional view these cells are exceptionally thin, and their elliptical nuclei, which stain intensely with Ehrlich's haematoxylin, are widely spaced. However, this wide spacing is not maintained in the region of the chorion, where the innermost nuclei are closely adjacent. The epithelial cells here probably have a smaller area than elsewhere. These cells are thickest in the regions of their nuclei, which are comparable in size with those of the mesenchyme cells.

The cells of the coelomic mesothelium constitute what is essentially a pavement epithelium, forming a continuous layer uninterrupted by intercellular spaces (Text-fig. 9). Each cell has a considerable surface area, being three, four, and sometimes five times as extensive as the endoderm cells. The cell outlines are extremely irregular and finely indented; there is very little intercellular substance present.



TEXT-FIG. 8. The endothelial cells of a branching vitelline vessel in surface view.



TEXT-FIG. 9. The coelomic mesothelium of the yolk-sac splanchnopleur at 24 days, in surface view, showing the outlines of an overlying vitelline vessel.

CONCLUSIONS

In sectional view the chorion at the 16-day stage is somewhat convoluted, these convolutions having generally disappeared in the 24-day stage. The dissection of late stages has revealed that the sinus terminalis is situated more antimesometrially in some swellings than in others. In some sections the mesenchyme, underlying the endoderm in the region between the sinus terminalis and the bilaminar tag, appears to have been under a tensile stress, which has subsequently been removed, permitting the imperfect contraction of the tissue and resulting in the wavy contour illustrated in Text-fig. 4. The increasing bulk of the developing embryo is catered for by the further growth and elasticity of the embryonic membranes. The membrane extending from the sinus terminalis to the peripheral region of the placenta probably permits of the more violent embryonic movements associated with late pregnancy, which result in the straightening of the chorionic ectoderm and the stretching, and subsequent imperfect contraction, of the mesenchyme of this zone. This would account for the differences in the relative position of the sinus terminalis.

The shortening of the bilaminar omphalopleur appears to be brought about by two concurrent processes. Firstly the degeneration at the free end, which was observed at both the 16- and 24-day stages. Secondly by a pulling apart of the endoderm and chorionic ectoderm. This second process is probably associated with increasing embryo size. At the 24th day the sinus terminalis is no longer proximate to the bilaminar tag, as in the 16-day stage, and squamous cells, similar to those of the endoderm of the bilaminar omphalopleur, are now present in the endodermal epithelium of the yolk-sac splanchnopleur. The mesenchyme underlying these spindle-shaped cells is of a type essentially similar to the mesoderm of the chorion, with which it appears to have a common origin. As the embryo enlarges the endoderm and chorion of the bilaminar omphalopleur are pulled apart, the process being accompanied by the further growth of mesoderm.

The area vasculosa is continually extending, but the pulling out of the bilaminar tag and the growth of mesoderm appear to be proceeding at a greater pace. This results in a non-vascular region becoming interpolated between the sinus terminalis and the base of the tag. Duval figures the sinus terminalis at the end of gestation near the base of the bilaminar omphalopleur which is now a very small papilla, the area vasculosa at term having completed its extension. The squamous cells of the yolk-sac splanchnopleur have thickened and are now cuboidal, being continuous round the end of the tag with the cuboidal cells of the ectodermal epithelium. A thin mesodermal wedge has penetrated between them; thus, the remnant of the bilaminar omphalopleur has, in fact, become trilaminar.

The distribution of the metaplasmic inclusions and mitochondria of the endodermal cells of the yolk-sac splanchnopleur has been described. In general, these substances are at their greatest concentration in the apical cytoplasm. Such a pattern of distribution, with the frequent basal position of the nucleus, suggests a secretory activity rather than an absorptive one. However, the nature of the folding and pleating of the epithelium is of a type which one generally associates with absorptive surfaces.

The mesenchyme layer of the yolk-sac splanchnopleur at the 24-day is more compact than at the 16-day stage, but it is still essentially embryonic in character; with the exception of the vitelline vessels it remains undifferentiated.

It has been shown (Brambell *et al.*, 1949) that circulating maternal antibodies pass readily into the foetal circulation in rabbits. This passage takes place exclusively by way of the uterine lumen, yolk-sac splanchnopleur, and vitelline circulation. When immune rabbit serum is introduced into the uterine lumen on the 24th day of gestation the passage of antibodies from it into the foetal blood can be demonstrated with regularity, but the passage of antibodies into the exocoelomic and amniotic cavities is extremely irregular and erratic. As a rule no antibodies can be detected in the exocoel or amnion after 24 hours, although by that time a relatively high titre is attained in the foetal blood: but, sporadically in some embryos, antibodies pass into the exocoel and amnion and can be detected at significant titres.

It is evident that the antibodies reach the foetal blood directly from the uterine lumen by traversing the intervening tissues of the yolk-sac splanchnopleur, and not by way of the exocoel. Hence they must first traverse the endoderm, then the splanchnic mesenchyme, and finally the capillary endothelium. Since the mesenchyme is an embryonic connective tissue, with tissue spaces in the matrix between the cells, it does not constitute a continuous cellular membrane as does the endodermal epithelium and the capillary endothelium. Thus it would seem probable that the selectivity displayed by the yolk-sac splanchnopleur in the free admission of rabbit antibodies, and the partial exclusion of bovine and equine antibodies (Brambell *et al.* (in press)), must be due to one or other, or both, of these membranes.

The route of entry of antibodies into the exocoel is by no means so clear. It is apparent from the description contained herein that antibodies could pass from the uterine lumen into the exocoel directly, either by traversing the yolk-sac splanchnopleur or the chorion. Since they must reach the mesenchyme of the splanchnopleur in their passage into the circulation, the only further barrier to their entry into the exocoel is the thin, squamous mesothelium lining the exocoel. This would seem the most likely route when a passage is effected, but since this is exceptional and their passage into the circulation is regular, it must be concluded that the exocoelomic mesothelium is relatively impermeable while the capillary endothelium is relatively permeable to them. Indirect passage from the foetal circulation into the exocoel can be ignored, since it would involve an identical route through the splanchnic mesenchyme and exocoelomic mesothelium.

Passage into the exocoel through the chorion, which is freely exposed to the uterine lumen, appears to be the only alternative. This would involve passage through the chorionic ectoderm, mesenchyme, and accessory exocoel, as well as the exocoelomic mesothelium, to reach the main exocoel. The accessory exocoel at the 16th day separates the multilayered chorionic ectoderm and its thin mesodermal lining from the inner vascular layer of mesoderm containing the vitello-allantoidean vessels. The accessory exocoel is not lined by a distinct mesothelium and it appears to have been produced as a split in the chorionic mesoderm which has grown down from the placental region. It does not appear to be in continuity with the cavity of the exocoel proper.

The accessory exocoel is reduced by the 24th day, the inner layer of mesoderm which has become almost non-vascular approximating to the inner surface of the chorion. The production of multinucleate spherules has almost ceased at this stage and, in parts, the chorionic ectoderm is composed of a single layer of cuboid cells.

The work was carried out during the tenure of an Agricultural Research Council studentship, and was financed by a grant from the Agricultural Research Council to Professor F. W. R. Brambell, F.R.S., in aid of his researches.

REFERENCES

BRAMBELL, F. W. R., HEMMINGS, W. A., HENDERSON, M., PARRY, H. J., and ROWLANDS, W. T., 1949. Proc. Roy. Soc. B, **136**, 131–44.

—— —— —— and ROWLANDS, W. T., 1950. Proc. Roy. Soc. B (in press).

BRANCA, A., 1923. Arch. de Biol., **33**, 605–70.

DUVAL, M., 1892. *Le Placenta des Rongeurs*, 77–119. Paris (Alcan).

SANSOM, G. S., 1927. Proc. Roy. Soc. B, **101**, 354–68.

The Effect of Inhibitory Substances on Cell Division

A Study on Living Cells in Tissue Cultures

By A. F. W. HUGHES

(*Sir Halley Stewart Research Fellow. From Strangeways Research Laboratory, Cambridge*)

With four Plates

SUMMARY

1. The effect of inhibitory substances upon cell divisions in chick tissue cultures has been studied mainly by phase-contrast ciné-micrography.

2. Fluoride and powerful -SH reactants prevent the entry of cells into prophase within 1 hour.

3. Aminopterin and colchicine are complete spindle inhibitors; they prevent its formation at the end of prophase, and can act on all metaphase cells in the culture.

4. The effect of fluoride, cyanide, and urethane on the spindle is much less drastic.

5. Most substances can retard nuclear reconstruction in telophase to some extent, but only chloracetophenone acts in the way already described for iodoacetamide.

6. Malonate appears to have no effect on mitosis, and fluoracetate only at very high concentrations.

7. Possible interpretations of some of these results are discussed.

CONTENTS

PAGE
INTRODUCTION . . . 252
MATERIAL AND METHODS . . . 252
NORMAL MITOSIS IN THE CHICK . . . 253
THE INHIBITORY AGENTS . . . 253
GENERAL EFFECTS ON DIVIDING CELLS . . . 254
SOME GENERAL TOXIC EFFECTS ON INTERPHASE CELLS . . . 255
DETAILED ACCOUNT OF OBSERVATIONS . . . 256
1. Colchicine . . . 256
2. Folic acid antagonists . . . 257
    (*a*) Aminopterin . . . 257
    (*b*) Aminomethopterin . . . 259
3. Sulphydryl reactants . . . 260
    (*a*) Chloracetophenone . . . 260
    (*b*) Iodoacetic acid . . . 263
    (*c*) Sodium selenite . . . 264
4. Sodium fluoride . . . 264
5. Hydrocyanic acid . . . 266
6. Urethane . . . 267
7. Amino-nitrogen mustard . . . 268
8. Sodium fluoracetate and sodium acetate . . . 269
9. Sodium malonate . . . 270
DISCUSSION . . . 270
REFERENCES . . . 274
DESCRIPTION OF PLATES . . . 276

[Quarterly Journal of Microscopical Science, Vol. 91, part 3, September 1950.]

INTRODUCTION

THIS paper describes the effect of a number of inhibitory substances on cell division in chick tissue cultures. It continues the work of Pomerat and Willmer (1939), who, in seeking evidence for the now outmoded thesis that glycolysis in the embryo is non-phosphorylating (Stickland, 1941; Barron, 1943; Novikoff, Potter, and Le Page, 1948), acquired a valuable series of results on the effects of enzyme inhibitors on the mitotic rate in living cultures.

This present work describes the results of phase-contrast ciné-micrography of living cells *in vitro* under the action of inhibitory substances. Such a study on the effects of the sulphydryl reactant iodoacetamide has already been published (Hughes, 1949). The observations on other substances recorded in this paper are also limited to the short term effects seen within 1–2 hours of the application of agents, which were frequently used at comparatively high concentrations. The results described are therefore restricted to one sector only of the range of action of mitotic poisons.

MATERIAL AND METHODS

The description of techniques given in my previous publication (Hughes, 1949) applies equally to the present paper. Explants of chick frontal bone are grown as hanging-drop cultures in chambers adapted both for phase-contrast microscopy and for irrigating the culture with saline while a cell in the outgrowth is being observed. An agent can thus be applied to a cell at any desired stage of mitosis. The behaviour of the cell is recorded by ciné-photography both before and after the application of the saline.

The substances under test were dissolved in Tyrode's saline, and the pH of the resulting solution was measured with Johnson's test paper No. 6883, and where necessary adjusted to within the range pH 7·4–7·7. Since most of the substances here investigated do not inhibit mitosis as rapidly as does iodoacetamide, the saline in which they were dissolved was left in contact with the culture throughout the experiment.

After the observation and cinematography of the cell had been continued for a sufficient period, the position of the cell in the outgrowth was carefully noted, and the culture was then fixed and stained, generally with Erhlich's haemoxylin. The same cell was identified in the permanent preparation and was compared with its appearance during life. The whole outgrowth of the culture was then studied to determine to what extent effects seen in the one cell were typical of other dividing cells.

As before, control observations were made by adding Tyrode's saline to the cultures, and following cells through mitosis. Again, the same cell was subsequently identified in the fixed and stained preparation.

The film records were analysed with the aid of a Craig 'Projecto-Editor' (see Fell and Hughes, 1949). To examine the effect of agents on the rate of cell division some experiments were made in which the culture, before and after treatment, was photographed serially at low power with the Canti apparatus in the way described by Fell and Hughes (1949). For the analysis of these films, I am indebted to my colleague Mr. J. Boss.

The chick cultures used in this present work were prepared by Mr. L. J. King, to whom once again I record my gratitude. The mouse cultures for certain experiments with chloracetophenone were made by Dr. H. B. Fell, to whom I am grateful for staining and mounting the cultures for some of these experiments. Miss J. Rawlinson has recently undertaken this work under Dr. Fell's direction.

NORMAL MITOSIS IN THE CHICK

The normal division of cells in living chick cultures has already been described by Hughes and Fell (1949) and Hughes (1949). The only period during mitosis when details are not clearly visible is during telophase, when the reconstruction of the daughter nuclei is in progress. Here comparison with the fixed and stained cell is most necessary.

Nuclear reconstruction in the chick is very similar to that in the mouse (Fell and Hughes, 1949). In late anaphase the groups of daughter chromosomes seem to fuse together, but often before cleavage is complete their appearance again changes, and a looser structure of the whole group is then observed. The nuclear membrane can then be traced as an envelope round the chromosomes which take on a ragged or 'fuzzy' appearance while they are losing their nucleoprotein charge. The area of the nucleus continually increases at this stage.

The nucleoli first appear about 12–15 minutes after anaphase began and at first are best seen in the living cell, as in the mouse. The last stages in the loss of stainable material from the chromosomes seem to proceed at a variable rate and 25–30 minutes after anaphase began the process is usually not quite complete. The young interphase nucleus then usually contains a number of stainable granules (Pl. IV, fig. 13), which are partly 'heterochromatic' chromocentres and residual euchromatin. These gradually become less distinct as interphase advances.

THE INHIBITORY AGENTS

The effects of these substances on dividing cells have been studied in this work:

1. Colchicine, the classical spindle inhibitor, with the action of which other substances were compared.
2. Competitive antagonists of pteroyl-glutamic (folic) acid aminopterin (4-aminopteroyl glutamic acid, Seeger *et al.*, 1947), and amino-methopterin (4-amino N^{10}-methyl pteroyl glutamic acid, Cosulich and Smith, 1948).
3. Sulphydryl reactants:
    Chloracetophenone (Dixon and Needham, 1946).
    Sodium iodoacetate.
    Sodium selenite.

Other substances:

4. Sodium fluoride (inhibitor of glycolysis, but specific only at low concentrations).
5. Hydrocyanic acid (inhibits cytochrome oxidase, but many other enzymes as well).
6. Urethane (a narcotic; inhibits some dehydrogenases).
7. Amino-nitrogen mustard (NN-di (2-chloroethyl) paraphenylene diamine; a vesicant and a 'radio-mimetic' poison).
8. Sodium fluoracetate (inhibits pyruvate metabolism and the cyclophorase system. Liebecq and Peters, 1949; Kalnitsky 1948).
9. Sodium malonate (inhibits succinic dehydrogenase).

GENERAL EFFECTS ON DIVIDING CELLS (see Table 1)

Under the conditions of experiment, the possible effects of inhibitory substances on cells about to enter or during mitosis are the following:

1. *Pre-prophase inhibition.* A cell may be prevented from entering prophase. While any substance added to the culture medium at an appropriate concentration would depress growth and mitosis, probably few agents will abolish prophases from a well-grown culture within an hour or so, and yet leave the interphase cells in a substantially normal state. In the present experiments, this was achieved by fluoride and some sulphydryl reactants only.

2. *Metaphase inhibition.* In general, if a cell has entered prophase in the presence of an inhibitor, the chromosomes form normally and the nucleoli and nuclear membrane disappear at the end of prophase, but the formation of the mitotic spindle may be suppressed. Thus, at appropriate concentrations, colchicine, aminopterin, and some sulphydryl reactants completely inhibit the development of the spindle. After 20 minutes or more of such treatment, early anaphases are absent or very rare in the outgrowth.

Other inhibitors, such as urethane, cyanide, and fluoride, allow the spindle to form, but metaphase may be prolonged; and anaphase is either delayed or suppressed. These less effective substances inhibit only a proportion of the cells in metaphase. Some proceed to anaphase, and early anaphases are found in the outgrowth after fixation and staining.

The spindle itself is not revealed by phase-contrast, but may be outlined as a granule-free area of cytoplasm, surrounded by cell inclusions. The effect of experimental conditions on the spindle, however, can be inferred from the behaviour of the chromosomes; the first sign of serious damage to the spindle is stoppage of the normal random movement of the chromosomes in metaphase (Hughes and Swann, 1948). I have never known a cell in which this movement has ceased to proceed to anaphase.

The next effect on cells in metaphase is 'clumping' of the chromosomes, which cease to be separately distinguishable. To assess the extent of this effect, it is necessary to compare the latter part of the film record of the cell during life with its appearance after fixation and staining.

Usually, if a cell is inhibited in metaphase, nothing further happens during the period of the experiment, and it is probable that eventually it would degenerate. Thus there is inhibition both of the spindle and of the reconstruction process through which the chromosomes normally pass after anaphase. Occasionally, however, in a cell inhibited in metaphase, the chromosomes reconstruct into a single nucleus, presumably a tetraploid. This can result on treatment with amino-nitrogen mustard or aminopterin (Pl. II, figs. 4 and 5).

In plant cells in which metaphase has been arrested by colchicine and other substances, it is well known that the metaphase chromosomes reconstruct as tetraploid nuclei. This has also been observed in animal cells by Gaulden and Carlson (1947) in grasshopper neuroblasts, and by Pincus and Waddington (1939) in rabbit eggs.

3. *Telophase inhibitors.* If anaphase once begins in the presence of an inhibitor usually it continues, though to this there are exceptions. Telophase, however, may be imperfect in two respects. The cleavage of the cell may be distorted or suppressed; also, the reconstruction of the daughter groups of chromosomes into nuclei may be retarded or impeded to a varying extent. Most substances at high concentration can retard nuclear reconstruction to some extent, but sulphydryl reactants may completely block the process, or at lower concentrations allow the nuclear membrane and the nucleoli to form, while retaining some chromosomes in almost their fully charged mitotic state. This effect with iodoacetamide has already been described (Hughes, 1949).

The features of the mitotic cycle, here most sensitive to the action of inhibitors, would therefore seem to be entry into prophase, formation of the spindle, cleavage, and reconstruction of the daughter nuclei. Mid-prophase and anaphase appear to be less sensitive.

SOME GENERAL TOXIC EFFECTS ON INTERPHASE CELLS

Although dividing cells are more sensitive to the effects of toxic substances than those in interphase, yet at the higher concentrations used, some effects common to both resting and dividing cells are sometimes seen. Miszurski (1949) has described bubbling over the surface of interphase cells with 10–0·001 mMolar colchicine. This is common with other agents, and probably can be induced by a wide range of treatments.

Small bubbles over the surface of the cell which are repeatedly extended and then withdrawn are readily distinguishable from the large irreversible blebs which herald the death of a cell after prolonged treatment with high concentrations of some agents. As these blebs are extruded, the nuclear membrane shrinks, and gradually the cells enters pycnosis. When fixed at this stage, such nuclei usually have a shrivelled appearance.

Well before this stage of damage is reached, there is another indication of injury which can best be revealed by time-lapse cinematography, namely, cessation of the normal movement of the intracellular inclusions (Lewis and

Lewis, 1924). This happens, for instance, after treatment for 30–40 minutes with iodoacetamide and chloracetophenone at the higher concentrations used; it is first seen in dividing cells, but later may affect the interphase cells also. When the normal and comparatively slow movement of the mitochondria has been stopped in this way, direct observation often shows that all the smaller granules in the cytoplasm are in Brownian movement which they do not normally exhibit. At this stage of injury to the cell, its appearance may still be normal after fixation and staining.

TABLE I. *Effects of Inhibitory Substances on Cell Division in Chick Tissue Cultures over a period of 1–2 hours, when applied for periods up to 1 hour at the concentrations indicated*

		Type and degree of inhibition		
Substance	*Concentration in mMols*	*Pre-prophase*	*Metaphase*	*Telophase (nuclear reconstruction)*
Colchicine	0·0027	None	Complete	None
Aminopterin	0·5	None	Complete	Slight–moderate
Amino-methopterin	5·5	None	Partial	None
Amino-N-Mustard	0·075	Little or none	Partial	None
NaF	10·15	Complete	Partial	Slight–complete
Urethane	84	None	Partial	Slight
HCN	5·10	None	Partial	Slight–moderate
CAP	0·065	Partial	Complete	Large
	0·33	Nearly complete	Complete	Complete
CH_2I COONa	0·033	Large	Partial	Slight
	1·8	Complete	Nearly complete	Moderate
Na_2SeO_3	1·0	None	Partial	Moderate
$CH_2FCOONa$	40	None	Slight	Very slight
Na malonate	≈70	None	None	None

An effect of poisons on the nucleus which is best seen in fixed cultures, is that the chromonemata may become abnormally distinct, sometimes to such an extent as to obliterate the visible distinction between cells in interphase and those in early prophase.

DETAILED ACCOUNT OF OBSERVATIONS

1. *Colchicine*, $C_{22}H_{25}NO_6$ (structural formula not yet certain; for possible formulae see Sexton, 1949, p. 342)

Among investigations into the effects of colchicine in tissue culture, those of Ludford (1936) and Verne and Vilter (1940) may be cited. To compare the effects of colchicine with those of other inhibitors which have been studied in the course of this work, 0·0027 mMolar colchicine (1:10^7) was added to four cultures and in them early prophases were found at 2–27 minutes afterwards, the subsequent fate of which was followed. In each instance, there was complete inhibition of the spindle, and no normal movements were seen in the chromosomes, which slowly clumped to a dense rounded mass (Pl. I, fig. 1). Bubbling was conspicuous over the cell surface and increased in extent as observation proceeded.

In six cultures fixed 40–60 minutes after the addition of 0·0027 mMolar colchicine, no anaphases were found, and in only one was a single late telophase to be seen.

Cells may enter division under the influence of colchicine, both in tissue culture and in the rat cornea, as was found by Buschke, Friedenwald, and Fleischmann (1943). This fact is illustrated by a low-power record of the part of the outgrowth of a tissue culture treated with 0·0027 mMolar colchicine. In the 5 hours preceding treatment, 25 cells divided. The agent was then added for 1 hour and then withdrawn; in the next 5-hour period, 20 cells entered prophase and rounded up in metaphase inhibition. Thus, there was little or no effect of the agent upon the entry of cells into prophase.

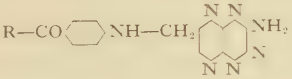
The lack of effect of 0·0027 mMolar colchicine on telophase in chick culture has already been described (Hughes, 1949).

2. *Folic Acid Antagonists*

Wagley and Morgan (1948) examined the effect of substances of this kind on the blood islands in the yolk-sac of the chick embryo. Several days after the agent had been injected into the yolk-sac cavity after treatment both with 4-amino, and 4-amino N^{10} methyl pteroylglutamic acid, nuclear degeneration was found in the blood islands which were smaller and less numerous than usual. The former substance (aminopterin) was about twice as effective as the latter (amino-methopterin) in causing this effect.

Thanks to the kindness of Dr. W. Jacobson, I have had the opportunity of testing the effect of these agents on cell division in chick tissue cultures. The substances were manufactured by the Lederle Laboratories of the American Cyanamide Co.

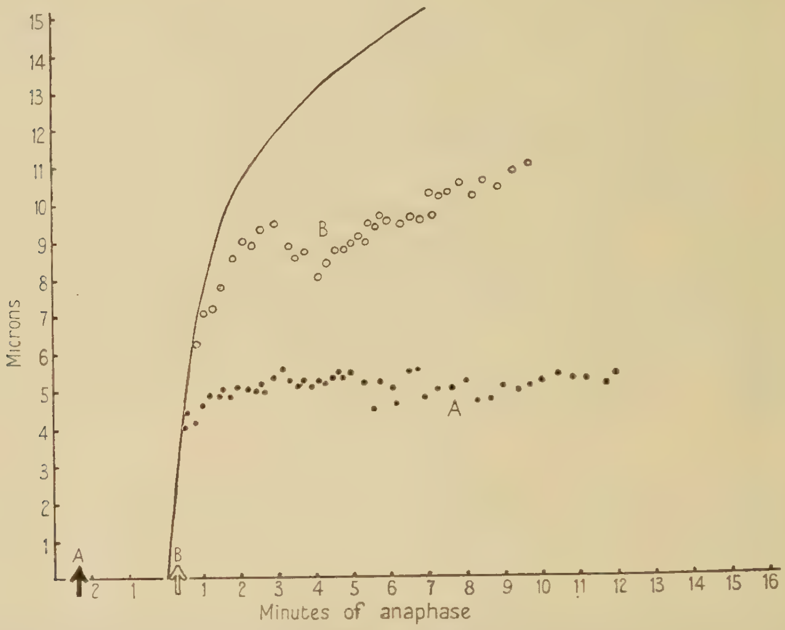
(*a*) *Aminopterin*,



(R = glutamic acid)

Aminopterin added to cultures at concentrations of 0·57–0·45 mMolar (1:4,000–5,000) acts as a complete spindle inhibitor. The effect on cells in early prophase was similar to that of colchicine, and anaphases became very rare or absent in cultures fixed after treatment for 8–25 minutes. In each of two cultures to which aminopterin at this concentration was added, a cell in early prophase was followed. The chromosomes formed, and the nucleoli and the nuclear membrane disappeared but no spindle developed. As with colchicine, the chromosomes gradually contracted into a compact group (Pl. I, fig. 2). In both, movement of the cytoplasmic granules continued. There was slight bubbling at the cell surface in one, and the cells gradually became rounded. In the other, the cell processes were not withdrawn, and there was no bubbling.

Two cultures were treated with 0·57 mMolar and one with 0·23 mMolar aminopterin; a cell in metaphase was followed in each for periods of 32–6 minutes. The spindle was inhibited and there was no subsequent anaphase.



Text-fig. 1. Anaphase curves of two cells in cultures treated with 0·45 mM aminopterin, compared with an average normal anaphase curve. Agent added (A) 2½ min. before, and (B) as soon as anaphase began. Nuclear reconstruction followed in (B) but not (A). Cleavage of the cell occurred in neither.

At the end of these experiments the appearance of the metaphase chromosomes varied between a closely packed group within a rounded cell, and a condition where they had begun to scatter throughout the cytoplasm. In one cell in metaphase treated with 0·57 mMolar aminopterin for 20 minutes, the chromosomes began to reconstruct as a tetraploid nucleus within an undivided cell (Pl. II, fig. 4). A nuclear membrane formed round most of the chromosomes. Such metaphase reconstructions were occasionally seen elsewhere in the outgrowth of cultures treated with this concentration of the agent.

In a culture treated with 0·45 mMolar aminopterin, a cell in metaphase proceeded to anaphase in the presence of the agent, but the anaphase movement of the chromosomes ceased after they had moved only 5 μ apart (Text-fig. 1, and Pl. II, fig. 6). No reconstruction or cleavage had occurred 18 minutes from the beginning of the experiment.

On two other occasions, when 0·45 mMolar aminopterin was added to a culture as soon as a selected cell had entered anaphase, separation of the chromosome groups ceased, and cleavage was prevented. In these cells, daughter nuclei were formed (Pl. II, fig. 7).

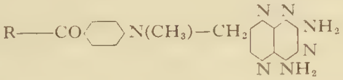
The effect of 0·45 mMolar aminopterin on reconstruction varies with the time in relation to anaphase at which it is applied. When the agent was added just after anaphase movement had begun in the cell under observation, cleavage and reconstruction proceeded almost normally, but granules of stainable material were seen along the chromonemata in the fixed and stained preparation. In the two other instances where the agent was added 2 or 3 minutes before anaphase began, the degree of inhibition in telophase was greater, as seen after fixing respectively 22 and 32 minutes from anaphase. The nuclear membrane formed in each daughter cell but the nucleoli were small or absent. The loss of material from the chromosomes themselves had not proceeded very far before fixation (Pl. IV, fig. 15).

The effect of aminopterin on reconstruction at half this concentration is very much less. Two cultures were treated with this agent at 0·23 mMols, and in each a cell in cleavage was found after 4–6 minutes. In one of these, cleavage proceeded normally with little more than the usual amount of bubbling. In the other, bubbling was abnormally profuse, and the two daughter cells had not effectively separated after 33 minutes. Reconstruction of the daughter nuclei was almost complete in each instance, the chromocentres being perhaps slightly more prominent than normal in the fixed and stained preparations.

A remarkable feature of the effects of aminopterin is that a substance capable of so profound an influence on dividing cells should exert little or no toxic effect on the culture generally. This is due to the fact that cultures spontaneously recover from its effects.

Some cultures treated with 0·45 mMolar aminopterin for 30 minutes showed appreciably more early anaphases than they did 10 minutes earlier. To study the course of recovery from aminopterin, six cultures were treated with the agent at this concentration for 38 minutes and after withdrawal of the solution they were fixed at varying intervals up to 2 hours afterwards. In all those fixed after a further 10 minutes, anaphases and telophases were plentiful. Nevertheless, inhibited metaphases do not disappear from treated cultures. Five hours after four cultures had been exposed for 1 hour to 0·23 mMolar aminopterin, arrested metaphases were common, and were still to be seen in cultures fixed 17 hours after treatment.

(*b*) *Aminomethopterin*,



(R = glutamic acid)

Aminomethopterin was applied in concentrations of 1·1–5·5 mMolar (1 : 2,000–1 : 400) to five cultures, in three of which cells in early prophase went normally through mitosis; only at the highest concentration was bubbling rather greater than normal during cleavage. In one culture treated with 2·8 mMolar aminomethopterin, the rapid movement of granules in the early daughter cells was conspicuous. In all the daughter nuclei, nucleoli and nuclear membranes formed normally; and examination after fixation and staining showed that the residual chromatin was not unusually prominent.

In two other instances, metaphase in a cell under observation was prolonged. In both these the spindle apparently formed normally, and random metaphase movement began. In one, this continued for 28 minutes, when the culture was fixed. At the higher concentration, random metaphase movement slowed down after 16 minutes and the chromosomes began to clump together: the culture was fixed 36 minutes later. In both cells there was continuous bubbling over the surface throughout the experiment, with consequent movement within the cell.

In cultures fixed 52 minutes after the application of 5·5 mMolar aminomethopterin there were many inhibited metaphases and anaphases throughout the outgrowth. Comparison of these two sets of observations suggests that, in chick tissue cultures, aminopterin is about twenty-four times as effective a spindle inhibitor as aminomethopterin.

The effect of pteroylglutamic acid itself on tissue cultures was tested. A saturated solution at 37° (< 5 mMolar) was applied for 57 minutes to three cultures, which were fixed at intervals up to 5 hours after treatment. The outgrowth of these cultures after fixation and staining appeared entirely normal.

3. *Sulphydryl Reactants*

There are interesting differences between the action of the four substances of this class which have been employed both in this and in the preceding paper (Hughes, 1949). Iodoacetamide and chloracetophenone behave similarly; both arrest movement within a cell and can block nuclear reconstruction completely, and both inhibit all metaphases at a concentration much less than that necessary to prevent cells entering prophase.

Iodoacetate is much more effective as a pre-prophase than as a metaphase inhibitor. Inhibition of nuclear reconstruction is only partial when comparatively high concentrations are added; and the motion of the granules and mitochondria in the cell is increased.

Selenite, at sub-lethal doses, only partially inhibits metaphase and nuclear reconstruction, and does not prevent the entry of cells into prophase.

No indication of nuclear reconstruction in inhibited metaphases was seen in cultures treated with sulphydryl reactants, though Beatty (1949) has shown that heteroploidy can result when frog gastrulae are treated with a concentration of chloracetophenone much below that which has been employed in the present work.

(*a*) *Chloracetophenone*, $\mathrm{CH_2Cl.\ CO}$

Prophase and metaphase. In six chick cultures treated with 0·65 and 0·33 mMolar CAP (1 : 10,000–20,000) cells in early prophase were identified and their subsequent fate was recorded. The normal disappearance of the nucleoli and the nuclear membrane was observed at periods of 5·7–12·4 minutes after the addition of the agent at the lower concentration, but no signs of the formation of a mitotic spindle were ever observed. At the higher concentration the latest time at which the nucleoli and nuclear membrane disappeared was 6·5 minutes after adding the CAP (Pl. I, fig. 3). The corresponding intervals with iodoacetamide were longer (Hughes, 1949).

In some cells treated with CAP, though not in all, there was slight bubbling over the surface when observation began. This gradually ceased, and the movement of the intracellular inclusions slowed down but normally had not ended after 25 minutes. In some instances it seemed that this inhibition of intracellular movement slowly spread to nearby interphase cells.

After the nucleoli had gone, the chromosomes slowly ceased to move. They never came into the metaphase position, nor did they fuse into the compact group seen after treatment with colchicine or aminopterin, but clumped into a continuous skein similar to that produced by iodoacetamide. In one instance, the chromosomes fused into three distinct knots.

Two prophases in mouse spleen cultures with 0·33 mMolar CAP behaved similarly, but in them granule movement ceased entirely after 20–5 minutes. In one, another prophase found after this period was completely inhibited; at the end of a further 40 minutes 'death blebs' were extruded from this cell.

In two instances where 0·33 mMolar CAP was added to chick cultures, cells apparently in prophase which were found a few minutes later failed to proceed with mitosis during the next half-hour. Owing to the difficulty of distinguishing between very early prophases and some intermitotic cells with conspicuous chromonemata, it is uncertain whether these are real instances of inhibition in early prophase.

CAP at concentrations of 0·33–0·065 mMolar was added to eight cultures. In each, cells in metaphase were filmed 1–2 minutes later for periods of 13–23 minutes. In every instance, random metaphase movement had ceased by the time the film record began, and in none was metaphase followed by anaphase. The effect of the agent on the metaphase chromosomes themselves was variable; sometimes they were unaffected and in fixed preparations such cells were indistinguishable from normal metaphases. In others there was slight clumping, which was more frequently, though not invariably, seen at the highest concentrations. Early anaphases were very rare in the outgrowth of cultures treated with CAP at concentrations of 0·065 mMolar or more.

The effect on the surface of cells in metaphase and on the movement of the cell granules was generally similar to that experienced with cells in prophase. Polar bubbling was common at the beginning of the experiment; often it was not more extensive than that over the surface of the nearby intermitotic cells. This gradually ceased during the following 15 minutes; movement of granules within the cell tended to decrease, though without ceasing entirely.

Anaphase and telophase. In each of a series of seventeen cultures, a cell was first observed in metaphase and watched until anaphase began. CAP at varying concentrations was then added to the culture and filming of the cell was begun as soon as possible, generally about ½ minute later. From the film record one could follow both the subsequent effect on the anaphase movement of the daughter chromosome groups, and the amount of distortion of cleavage; the corresponding fixed preparation showed the extent to which nuclear reconstruction was inhibited.

Anaphase curves were plotted from thirteen such records. At concentrations of 0·033 mMolar or more, the rate of movement of the chromosomes was always less than normal. At concentrations of 0·13 mMolar or more, movement had stopped by the time the film record began. Hence CAP at this concentration was able in about ½ minute to penetrate both clot and cell-wall, and build up a concentration within the cell sufficient to stop anaphase movement. As soon as it was certain that anaphase had begun, the daughter groups were about 4 μ apart. When the film records began this distance had increased to 7–8 μ. In the normal curve of anaphase in the chick (Hughes and Swann, 1948) separation of the chromosome groups from 4 μ to 7 μ occupies about ½ minute. This suggests that when 0·13 mMolar CAP is added to a culture as soon as anaphase begins in a cell, the movement of chromosomes continues until the inhibitory concentration is reached and then abruptly ceases.

Only at the lower concentrations (0·013–0·033 mMolar) where anaphase movement was merely slowed down, did anything like a normal cell cleavage occur, though this was accompanied by a marked excess of bubbling. Usually the flattening of the daughter cells was inhibited, with continual bubbling over the cell surface.

At the higher concentrations a regular division furrow never appeared, but a little later than the time when cleavage normally begins bubbling at the cell surface increased in violence to such an extent that the whole cell was fragmented into blebs connected by protoplasmic strands, in one of which the daughter chromosome groups were found quite unchanged, still in their early anaphase relation (Pl. IV, fig. 17). The slow development of this turmoil at the cell surface is in contrast to the rapid inhibition of anaphase movement of the chromosomes within the cell. The activity of the cell gradually slowed down and stopped entirely 10–15 minutes after the application of the agent. In some cells cleavage was distorted in other ways. The two poles of the cell could draw apart with such violence that the daughter chromosome groups were separated, and each was then drawn out into an elongated skein. Otherwise, both might remain at one end of the cell. Sometimes the cytoplasm was separated into three portions, two of which contained a group of chromosomes.

At a concentration of 0·065 mMolar at which anaphase movement was not completely arrested, cleavage was much distorted, but amid the turmoil in the cell, some degree of nuclear reconstruction took place. Thirty minutes after the application of the CAP, such partially inhibited daughter nuclei had usually formed a nuclear membrane, which enlarged to some extent, but the chromosomes were largely retained, and the nucleoli were either small or absent. With 0·033 mMolar CAP, nucleoli and nuclear membranes were usually of normal size, but some chromosomes were retained in the manner already described for iodoacetamide (Hughes, 1949). This condition, which is distinct from a mere retardation of reconstruction, is seen only with –SH reactants, and may be considered a specific effect of these agents on nuclear reconstruction.

(*b*) *Iodoacetic acid* (*IAA*)

Pomerat and Willmer (1939) found that 0·05 mMolar iodoacetate was sufficient to inhibit cell division in their cultures after 2 hours; my results showed that in hanging-drop cultures treated for 45 minutes with concentrations of IAA of the same order, prophases were very rare. On the other hand, anaphases were still occasionally seen in cultures treated for the same time with 1·8 mMolar IAA.

1·8–0·54 mMolar IAA (1 : 3,000–10,000) was added to five cultures and in each a cell in metaphase was selected for observation. The spindle always remained unimpaired, though metaphase was sometimes prolonged; the interval between the first addition of the agent and the beginning of anaphase was 5–27 minutes. Anaphase was entirely normal, and cleavage was accompanied by little more than the normal amount of bubbling. In one instance, the cleavage was asymmetrical, and in two others the daughter cells fused together again afterwards. The activity of the intracellular granules was markedly greater than normal.

Iodoacetic acid at the higher concentration of 5·4 mMolar (1 : 1,000) was added to five cultures, and in each a cell was selected for observation, to see whether metaphase would be arrested under these conditions. The cultures were fixed 21–40 minutes after the IAA had been applied. None of these cells proceeded to anaphase, although in one, 11 minutes after the addition of the agent, the chromosomes separated irregularly, and possibly were arrested in early anaphase. In all of these examples the random metaphase movement of the chromosomes ceased; in four, it ended within 1–5 minutes of the application of the agent, but in one it persisted until 8 minutes afterwards. Movement of the intracellular granules was observed in most; in one there was slight bubbling at the cell surface, which ceased 12 minutes after the IAA was first applied. Early anaphases were rare but not absent in cultures treated with 5·4 mMolar IAA for 25–40 minutes.

In cultures fixed and stained after treatment with IAA at the higher concentrations, the chromosomes of inhibited metaphases are often scattered throughout the cell. This is probably due to the increase in normal intracellular movement which is caused by IAA. It is not impossible that some degree of chromosome fragmentation may occur, for iodoacetic acid has vesicant properties (Bacq, Goffart, and Angenot, 1940).

In three cultures 5·4 mMolar IAA was added at the moment when a cell under observation entered anaphase. Each cell continued mitosis; cleavage proceeded almost normally with little if any more bubbling than in untreated cells. Flattening of the daughter cells was partially inhibited in one instance, and in another the daughter cells remained connected by a broad asymmetrical bridge. Examination of these three cultures after fixation and staining showed that nuclear reconstruction was inhibited to the extent that the nucleoli were either absent or very small (Pl. IV, fig. 16). The nuclear membrane had formed round each daughter group, and had enlarged considerably. There was a good deal of chromosome retention of the same order as that observed with 0·065 mMolar CAP, i.e. at about 1/100 of the concentration of IAA needed to produce the same effect.

At the lowest concentrations of IAA (5·4 mM.) the daughter nuclei in these cells which had undergone mitosis under observation showed only a slight retention of chromosomes, and the nucleoli were almost normal in appearance. The effect of 1·8 mMolar IAA to telophase under these conditions was intermediate between that of the higher and lower concentrations.

(*c*) *Sodium Selenite*

10–0·01 mMolar Na_2SeO_3 was applied to thirteen cultures. After 30 minutes' treatment with 10 mMolar selenite, most of the cells in the outgrowth of these cultures were rounded and blebbed; of six cultures treated with 1 mMolar selenite for 40–50 minutes, two after fixation and staining showed a large area in which the nuclei were shrivelled. At this concentration, cells were not prevented from entering prophase and the metaphase inhibition was only partial. With 0·01 mMolar selenite, some cells in metaphase arrest were seen in one culture after 43 minutes.

In each of four cultures treated with 1 mMolar selenite, the behaviour of a cell during mitosis was recorded. One was inhibited in metaphase, the other three were followed from metaphase through anaphase and cleavage. In the first cell, prophase proceeded normally and chromosome movements were seen after the nuclear membrane and nucleoli had gone. These movements continued, but the chromosomes never formed a regular metaphase plate, but became arranged in a ring. There was little or no clumping, and movement of the cytoplasmic granules was active, but there was no bubbling at the cell surface. In the three cells which were followed from metaphase onwards, a normal anaphase and cleavage occurred and the daughter cells flattened almost normally. Reconstruction of the daughter nuclei, however, was partially inhibited (Pl. IV, fig. 14). Chromosomes were retained to a variable extent, and in one pair of daughter cells the nucleoli could not be recognized with certainty.

4. *Sodium Fluoride*

Pomerat and Willmer (1939) found that under the conditions of their experiments, 20 mMolar fluoride was lethal to tissue cultures almost at once, while at 10 mMols, cell division stopped within 2 hours, though the culture itself survived for a further 10 hours or more. In the present experiments in cultures treated with fluoride at concentrations of 10–15 mMols, prophases completely disappeared from the outgrowth after 40 minutes, but anaphases, some of them much distorted, were then still seen. Inhibition of mitosis in cultures with fluoride as with iodoacetate is thus due to prevention of cells from entering prophase.

Study of living cells in the presence of fluoride shows this agent to be only a partial inhibitor of the mitotic spindle. Fifteen mMolar fluoride was added to a culture, and 17 minutes later a cell in prophase was selected for observation. The nucleoli and nuclear membrane vanished after a further 10 minutes and during the next 7 minutes the mitotic spindle formed rather slowly; random metaphase movement of the chromosomes then began and persisted till the cell was fixed 25 minutes later, although there was some clumping of the chromosomes. The spindle was clearly outlined in the cell during life. In another culture which had been exposed to 10 mMolar fluoride for about 30 minutes, a cell was followed from prophase; again the spindle formed normally and the cell remained in metaphase for 40 minutes before fixation.

When a cell was fixed after a prolonged metaphase in the presence of fluoride, it was not possible to decide whether anaphase would ultimately have occurred. In two other instances where the record began in metaphase, the cell remained in this phase for a further period of 43–9 minutes respectively. In a series of cultures treated with 10–15 mMolar fluoride, in which cells in metaphase were selected for observation, anaphase occurred at the following intervals after the first addition of the agent—8, 8, 11, 26, 33, 50, and 78 minutes.

Some bubbling at the surface of cells in metaphase was observed, but this was often no more than the usual amount during cleavage. Lagging of chromosomes was common in early anaphase, but in the examples seen during life, the delayed chromosomes always caught up with the others before cleavage. However, in one fixed culture, micronuclei were seen in several daughter cells, suggesting that lagging chromosomes had reconstructed individually, as in the experiments of Hughes and Fell (1949) on mustard gas.

Cleavage was often much delayed. In one example (Pl. III, fig. 9) the cell elongated after metaphase until the daughter chromosome groups were 16μ apart. Finally, the cell divided 20 minutes after anaphase began.

The degree of inhibition in telophase was apparently proportional to the time during which in metaphase the cell was under the influence of the fluoride, and varied from a very slight retention of chromosomes with flattening of the daughter cells (8 minutes) to a complete inhibition of flattening and reconstruction (78 minutes).

Partial Recovery after Fluoride. Since it has been shown that the effects of fluoride on the inhibition of glycolysis can be reversed by washing with fresh Ringer (Ewig, 1929; Kisch, 1934), it was decided to examine the effects of this procedure on tissue cultures which had previously been exposed to fluoride.

In three cultures treated for 60–70 minutes with 10 mMolar fluoride and fixed 64–90 minutes after withdrawal of the agent, prophases reappeared. In order to see whether further recovery was possible, each of a series of cultures treated for 40–55 minutes with 15 mMolar fluoride was subsequently left in contact for the same period with one of the following saline solutions:

(1) Plain Tyrode (which is 2 mMolar for Ca and 0·5 mMolar for Mg).
(2) Tyrode with 12 mMolar Ca.
(3) Tyrode with 10·5 mMolar Mg.
(4) Tyrode without Magnesium.

In group (1), during the 4 hours after removal of the Tyrode, all stages of mitosis reappeared in large numbers in four cultures, but in three others there was little or no recovery. Obviously inhibited metaphases remained conspicuous in all, and many degenerating cells were seen in the outgrowths. Essentially the same result was obtained with Tyrode containing extra calcium; extra magnesium under these conditions was definitely toxic, however, and in 3 hours the treated cultures were quite degenerate. There was some recovery in group (4), but inhibited metaphases were more conspicuous than with normal Tyrode.

In two cultures treated with 15 mMolar fluoride and subsequently with Tyrode, a metaphase was followed through the rest of mitosis; in one the Tyrode was without magnesium. The two cells in metaphase were selected 125 and 153 minutes respectively after the withdrawal of the Tyrode. In both a normal anaphase and cleavage took place, and only a small degree of chromosome retention was seen in the daughter nuclei after fixation, 23 and 29 minutes respectively after anaphase began.

Two other cultures were treated with 15 mMolar fluoride for 37 minutes; 14 minutes after withdrawal a metaphase was found in one, and was followed for 40 minutes through anaphase and a very distorted and bubbly cleavage. Tyrode was then added to the culture, and filming was continued for a further 34 minutes, when the culture was fixed. The daughter cells had flattened; in one a nucleolus had formed, but in the other cell the nucleus was in two halves with only very small nucleoli. The degree of chromosome retention was much less than would have been expected in the absence of the Tyrode. In the other culture, Tyrode was added 90 minutes after withdrawal of the fluoride and an obviously inhibited metaphase was followed for a further 20 minutes, during which the cells remained unchanged.

Therefore, although a partial recovery of the culture as a whole was demonstrated, the question remains open to what extent the individual inhibited cells can recover and complete their mitosis. It is probable that a large proportion of such cells would degenerate.

5. *Hydrocyanic Acid*

Pomerat and Willmer (1939) found that only with a concentration of 2 mMolar cyanide was any appreciable effect on cell division experienced under their conditions, and that even at this concentration cells were still dividing 10 hours after the substance was added. Using cyanide at concentrations of 1–10 mMolar I have found that at the upper limit of this range, both prophases and anaphases are still present in cultures after an hour's treatment. One cell under the influence of 1 mMolar cyanide was followed from early prophase through an almost normal mitosis. It remained in metaphase for 23 minutes, was rather more rounded than usual, and the daughter cells only partially flattened out at telophase.

Cells in metaphase were observed in each of ten cultures to which 5–10 mMolar cyanide was added; three were inhibited in metaphase for periods of 40–52 minutes, six proceeded to anaphase, and in one there was an abortive anaphase 17 minutes from this point. Of the three cells which were inhibited in metaphase, two were in the radial stage when the record began. Of these, one with 5 mMolar cyanide stayed for 14 minutes in this stage and then came into full metaphase; the other, with a concentration of 10 mMolar, stayed in the radial stage for the whole duration of the experiment, namely, 40 minutes. Generally, movement of granules ceased in cells, under the influence of cyanide, both in inhibited metaphases and in those which proceeded to reconstruction. This movement ceased sooner in mitotic cells than in adjacent interphases, but has been seen to stop also in the latter. In one metaphase arrest with 5 mMolar cyanide, 'death blebs' were extruded at the end of the sequence.

With cyanide treatment there was never a great exuberance of bubbling of cells in cleavage. There was the usual tendency to inhibit flattening in early interphase.

Inhibition of reconstruction varied from a very slight retention of chromosomes in otherwise normal daughter nuclei, to a more severe condition in which the nucleoli were rather small and dense. Three cells were observed to enter anaphase in the presence of 10 mMolar cyanide. In one, in which anaphase began 4 minutes after the addition of the agent, there was slight retention of chromosomes in the daughter nuclei; in the other two, where anaphase began respectively 6–18 minutes after this point, nuclear reconstruction was inhibited to a greater extent (Pl. III, fig. 10).

One culture treated with 10 mMolar cyanide for 64 minutes and fixed 376 minutes later, showed a few clumped metaphases with two late telophase stages, suggesting that inhibition of metaphase was even then not complete. In this culture the intermitotic cells showed no signs of pycnosis; they were rather larger than normal, presumably due to intake of water; one was filmed for 15 minutes and showed an abnormally active movement of the cytoplasmic inclusions.

6. *Urethane*, $NH_2.CO.OC_2H_5$

The effect of urethane on cells in tissue culture has been studied by Bastrup-Madsen (1949), who has described the arrest of cells in metaphase by this agent, and by Lasnitzski (1949) who has compared its action on a number of tissues, both embryonic and malignant. I have found that when 84 mMolar (1:100) urethane was applied to cultures for periods of up to 1 hour, some cells were arrested in metaphase, but that in others mitosis proceeded to anaphase and telophase. Anaphases were numerous in the outgrowth of cultures fixed after 1 hour's treatment.

Urethane at this concentration was applied to three cultures, and 4–7 minutes later in each a cell in prophase was found and followed. One went normally through mitosis, the other two were inhibited in metaphase for 24–37 minutes. In all, the disappearance of nucleoli and of the nuclear membrane proceeded normally, and was followed by a period of several minutes in which the chromosomes were actively moving, but were not arranged in a metaphase plate. The cell which proceeded to anaphase was the first of these to achieve the normal metaphase arrangement and movement of the chromosomes. In the other two, movement of the chromosomes was terminated by their clumping into dense groups, one of which resembled a metaphase plate. At fixation each of these cells was small and rounded. The appearance of the apparently inhibited metaphases in the fixed cultures varied. In some the chromosomes were tightly clumped, but in others a few of the chromosomes were loosely scattered throughout the spindle area.

There were small bubbles at the cell surface from the beginning of the record in both cells which were arrested in metaphase. In one the extent of the bubbling increased with time. In the cell which underwent cleavage, the degree of bubbling during that process was very great, and was sufficient to obscure the division of the cell until the two daughters had separated. Bubbling continued during the rest of the record, and no flattening of the daughter cells took place. In the daughter nuclei reconstruction was slightly retarded. Nucleoli and nuclear membranes were formed, but the loss of stainable material from the chromosomes was delayed.

After this treatment with 84 mMolar urethane, prophases were still seen in fixed cultures. In one experiment part of the outgrowth of a culture was photographed at low power for 5 hours. Then 84 mMolar urethane in Tyrode was added and photography was continued. After a further 4 hours, the urethane-Tyrode was withdrawn. During the first 5 hours of this record, twenty cells completed division. In the next 5 hours the same number of cells rounded up as inhibited metaphases, and did not proceed to cleavage. This suggests that the rate of entry of cells into prophase was unaffected by the treatment.

7. *Amino-nitrogen Mustard*,

$\mathrm{NH_2}\langle\;\rangle\mathrm{N}\begin{matrix}\mathrm{CH_2CH_2Cl}\\ \mathrm{CH_2CH_2Cl}\end{matrix}$

The effect of this substance upon growth and division in chick tissue cultures is being studied by Dr. Rinaldini, who will report upon his findings in due course. Below are described the results of some experiments on this substance, which were made in collaboration with Dr. Rinaldini.

The hydrochloride of this substance (ANM) was applied to thirteen tissue cultures at concentrations of 0·0037–0·075 mMolar ($1:10^7$–$1:5\times10^4$), and cells in various phases of division were followed, either soon after application of the agent or within 2 hours after its withdrawal. Under these conditions ANM behaved as a partial spindle inhibitor, and did not show any 'radiomimetic' effects. No immediate effects were seen at the highest dilution employed; a cell was followed from prophase through a normal mitosis in a culture exposed to this concentration. However, in four cultures treated with 0·037 mMolar ANM, in each of which a cell in metaphase was observed after the addition of the agent and followed through anaphase, cleavage was imperfect in three instances; either it did not occur or was distorted, or the daughter cells reunited. After 35–50 minutes' treatment of cultures with ANM at this concentration, inhibited metaphases were conspicuous.

In order to prolong the duration of these experiments, seven cultures were treated with 0·075–0·037 mMolar ANM for 15 minutes, and cells were subsequently observed in them. In three cultures, at intervals of 2–15 minutes after this treatment, a cell in prophase was followed. In two, mitosis proceeded normally, but in the other two the cell was inhibited in metaphase for 28–66 minutes.

In the three remaining cultures, cells in metaphase were followed for 61–74 minutes after treatment, One in which the spindle was present continued normal mitosis, but in the other two, which were apparently without a spindle, the metaphase chromosomes reconstructed into a single large nucleus, and the cell entered interphase as a tetraploid (Pl. II, fig. 5).

When the cultures had been fixed and stained, those daughter cells which had undergone a normal mitosis were examined to see whether the agent exerted any effect on nuclear reconstruction. In each instance, the daughter nuclei appeared entirely normal (Pl. IV, fig. 12).

8. *Sodium Fluoracetate*, $CH_2F.COONa$, *and Sodium Acetate*

Allsop and Fell (in the press) found that approx. 1·5 mMolar fluoracetic acid, in the medium of chick heart cultures, had little or no effect on their growth. I have applied sodium fluoracetate (Fl-Ac) to cultures at much higher concentrations, and can confirm the observations of these authors.

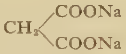
Two cultures were treated with 2 mMolar Fl-Ac. Sixteen minutes later, a cell in prophase in each was followed through an entirely normal mitosis. The same result was obtained with 10 mMolar and even with 40 mMolar Fl-Ac. When the cultures had been fixed and stained, it was noticed that the residual chromatin in the daughter nuclei was no more prominent than in untreated cells at the same stage (Pl. III, fig. 11, and Pl. IV, fig. 13). However, later in interphase there seemed to be no further decrease in amount of the residual chromatin, and sister cells could be traced in the outgrowth of such a culture, when they had already migrated some distance away from each other. In the outgrowth of these cultures cells in all stages of mitosis could be seen. There were some inhibited metaphases, but fewer than normal anaphases and telophases.

The next experiment was to use an even higher concentration of Fl-Ac, and to compare its effects with those of sodium acetate itself at the same concentration. Three cultures were treated with 100 mMolar Fl-Ac in Tyrode. This solution was appreciably hypertonic. In one culture a cell in late prophase was followed; the chromosomes clumped together soon after the nuclear membrane had gone, though there was some slight random movement at first. Movement of the granules was maintained within the cytoplasm.

These results were then compared with the effects of sodium acetate. Three cultures were treated with 100 mMolar sodium acetate in Tyrode for periods of 37–63 minutes, and in two of them a cell in prophase was followed to a similarly arrested metaphase. Comparison of the fixed and stained cultures previously treated with the two agents at this concentration showed that their effects were hardly distinguishable. There were no anaphases in the cultures treated with Fl-Ac, or in two of those treated with acetate. Nearly all metaphases were obviously inhibited, with the chromosomes entirely clumped together. There was evidence of the retention of chromosomes in telophase in both sets. Few or no cells were seen in late prophase, but it was not possible to judge whether the entry of cells into prophase had been entirely prevented, because the chromonemata in many interphase cells were abnormally distinct, and were indistinguishable from very early prophase chromosomes.

Since the results of treatment with both Fl-Ac and acetate at this high concentration are so similar, it cannot be claimed that any specific effect of fluoracetate has here been demonstrated.

9. *Sodium Malonate*,



$$CH_2\begin{matrix}\diagup COONa \\ \diagdown COONa\end{matrix}$$

Pomerat and Willmer (1939) found that 0·01 Molar sodium malonate had no effect on the growth of a culture over a period of 12 hours. In order to increase further the concentration of malonate, a modification of Tyrode's saline was prepared with 0·065 Molar sodium malonate and a reduced NaCl concentration of 0·073M. This was applied to three cultures for periods of 60–75 minutes, and no influence on cells in division was observed either during or immediately after the application. There were no effects to be seen in the outgrowth of the culture after fixation and staining, and the number of cells in each stage of mitosis was in the normal proportion.

A Tyrode was then prepared in which all the sodium was present as malonate (0·138 Molar; 17·4 gr. per litre). Embryo extract was prepared with this in the usual way, and a series of six tissue cultures were made, in which the medium was composed of 1 drop of malonate extract and 1 drop of plasma. The two sets of cultures were examined after fixation and staining. There was little or no difference between the growth and mitosis of these cultures and a series of controls prepared at the same time with extract prepared with normal Tyrode. I am grateful to Mr. L. J. King for performing this experiment for me.

DISCUSSION

P. Dustin (1947, 1948, 1949) has distinguished between two types of mitotic poisons, the spindle inhibitors such as colchicine, and the 'radiomimetic' poisons such as trypoflavin which, when injected into an animal, cause dividing cells to degenerate. Dustin has pointed out that the distinction between the two types of action is not clear cut. Recently, the effect of radiomimetic poisons in causing breakage of chromosomes has received attention (Darlington and Koller, 1947; Hughes and Fell, 1949; Loveless and Revell, 1949). Mainly owing to the short duration of the experimental period, the present observations are not concerned with radiomimetic effects, though the degeneration in arrested metaphases following treatment by fluoride and the possible fragmentation of chromosomes by iodoacetate are related to this type of action. Amino-nitrogen mustard under the present conditions also behaves merely as a spindle inhibitor.

The basic aim of research on mitotic poisons, and indeed in all experimental pharmacology, is to relate visible cell and tissue changes to biochemical events within the cell. Dustin in 1947 suggested that mitotic poisons are enzyme inhibitors. While it is to be expected that treatment of a cell with toxic substances at sub-lethal doses will affect its enzyme systems, it is by no means easy to relate the results of studies on isolated enzyme systems with experimental effects in whole cells. The study of the action of narcotics is concerned with the same general problem, as is ably shown by a recent review (McElroy, 1947).

In general, an initial difficulty in comparing the effects of inhibitors on isolated enzyme systems and on whole cells is that the sites of action of the enzymes are unknown, and that the permeability of the cell wall to the agent can only be surmised from general principles. Consequently, comparison of the concentrations effective *in vivo* and *in vitro* is not possible. The subject of permeability and drug action has recently been discussed by Trim and Alexander (1949). There is evidence that some enzymes are located at cell surfaces; Rothstein and Meier (1948) have shown that ATPase is present at the surface of the yeast cell, and both Willbrandt (1938) and Davson (1941) have indirect evidence that some glycolysis in the erythrocyte occurs at the cell curface.

The possible complexity of the effects of an inhibitor on the living cell is well illustrated by the action of fluoride. In isolated enzyme systems, as is well known, fluoride acts by removing magnesium, as in the classical instance of enolase (Warburg and Christian, 1941) which is the point of action at which fluoride inhibits glycolysis. However, in the intact cell, fluoride may remove calcium (Borei, 1945), and also allow potassium to escape from the cell surface, as Davson (1941) has shown for the mammalian erythrocyte, the normal K content of which is maintained by some energy-liberating process, such as glycolysis which is inhibited either by fluoride or iodoacetate.

Further, Davson and Danielli (1938) have shown that in general potassium escapes from the erythrocyte under 'pre-haemolytic' treatment, that is treatment by an agent which in higher concentration causes haemolysis. Hypotony can also be pre-haemolytic. These results can be compared with some that have emerged during this present work. Three pre-haemolytic agents, fluoride, iodoacetate, and, as will be shown in a succeeding paper, hypotonic saline, can readily prevent cells from entering into prophase. Moreover, cyanide and urethane, which are not pre-haemolytic, are much less effective as pre-prophase inhibitors in chick cultures. Two potent spindle inhibitors, colchicine and aminopterin, do not prevent the entry of cells into prophase under the conditions of the present experiments. It would be interesting to know whether they can act as pre-haemolytic agents.

The attempt to relate inhibition of the spindle to inactivation of enzyme systems seems about the most difficult task that could be attempted in the whole field, for the following reasons:

1. Spindle inhibition can be brought about by a very wide range of substances.
2. Of these the best known, namely, colchicine, exercises a range of other effects on cells and tissues which do not seem amenable at present to a comprehensive explanation.

The analogy between spindle inhibition and narcosis, to which Levan and Östergren (1943) and Östergren (1944) have drawn attention, at first seems to be applicable only to lipoid-soluble substances which these authors have shown to cause 'C mitosis' in *Allium* roots, and which, as Dustin (1947) has already pointed out, has apparently no relevance to other spindle inhibitors. However, McElroy (1947) in his recent review, to which reference has already been made, develops the thesis that the fundamental action of narcotics is to combine non-competitively with the protein portions of enzymes, and to cause their reversible denaturation, and that 'other theories of narcosis which consider such physical properties as partition coefficient, surface activity, vapour pressure, and water solubility can be related thermodynamically to the above theory of protein denaturation'.

McElroy's thesis offers some hope of extending the narcosis of spindle inhibition to include most instances of metaphase arrest by non-specific substances, which are usually effective only at comparatively high concentrations. However, Dustin's point remains that colchicine cannot be fitted into such a scheme, and is emphasized by comparison of the effects of urethane and colchicine on mitosis in the chick, which have been described in the preceding pages. Colchicine, however, does influence the physical properties of cytoplasm, as is shown by the experiments of Beams and Evans (1939), who found that the fertilized eggs of *Arbacia punctulata* were more readily stratified by centrifugation after treatment with colchicine.

Aminopterin is another substance of almost equal potency with colchicine as a spindle inhibitor. It is the most powerful competitive antagonist for folic acid yet prepared. Very little is yet known about the role played by folic acid within the cell; most of the numerous studies on the effects of folic acid antagonists are concerned with their inhibition of growth in tumours (Meyer, 1948; Moore *et al.*, 1949) or on bacterial cultures. Stokstad *et al.* (1948) believed that folic acid is here concerned with the synthesis of purines and pyrimidines. However, the rapidity of the effect of aminopterin on cells in mitosis points to its interference with processes which are not necessarily synthetic reactions. This substance is able to inhibit anaphase movement within 3 minutes of its addition to a culture; some immediate effect on an enzyme system presumably must occur. One enzyme that has so far been shown to be inhibited by a folic acid antagonist is dopadecarboxylase (Martin and Beiler, 1947), though similar observations on other systems may be expected.

Dustin has suggested that 'all mitotic poisons appear to share in common the property of inhibiting the enzymatic metabolism of carbohydrates'. Bullough (1949) has shown by elegant means that in the intact mouse a certain concentration of glucose is necessary to enable cells to enter prophase, but that mitosis can continue in its absence. This fact may well be related to the action of fluoride and iodoacetate which are inhibitors of glycolysis in preventing cells from entering prophase. However, Bullough's results indicate that aerobic conditions are also necessary for cells to enter division in the intact mouse, which may not be true for cultures of embryonic tissue, if the apparent lack of effect of fluoracetate on them is significant. Allsop and Fell (in the press) found that embryonic heart cultures cease to beat in the presence of one-hundredth the concentration of fluoracetate which in my experiments was necessary to stop mitosis. This result suggests that the energy level required for such a cell as a fibroblast to divide may be low, and recalls the observation of Burrows (1921) that the 5-day chick heart is able to grow anerobically in culture. The dividing chick cell seems insensitive to malonate and relatively insensitive to cyanide; although the enzymes inhibited respectively by these agents, namely, succinic dehydrogenase and cytochrome oxidase, are, according to Albaum, Novikoff, and Ogur (1946), both present in the chick embryo at a constant ratio from the fifth day to the end of incubation. The effect of cyanide in high concentration on mitosis may perhaps be related to the effect described by Albaum, Tepperman, and Bodansky (1946) on brain tissue in which cyanide poisoning was shown to reduce the level of high-energy phosphorus compounds.

We must now discuss some possible interpretations of the results in this and the preceding paper, of the action of sulphydryl reactants upon mitosis. It is hardly to be expected that there would be a single mode of action in the separate inhibitions in pre-prophase, metaphase, and telophase. Possible explanations fall into two classes, namely, action of the reactant on either enzymes or substrates.

The effect on the spindle may be due to the reaction with –SH groups at the surface of the constituent proteins. The inhibition of nuclear reconstruction at telophase is probably a more complex effect. Although it is seen after treatment with substances other than sulphydryl reactants, yet with them it seems to be a general retardation of the whole process. Only with sulphydryl reactants are apparently whole chromosomes retained in an otherwise normal daughter nucleus. This effect may be due to reaction of the agent both with intranuclear nucleoproteins and also with enzymes in cytoplasm and nucleus which affect the chromosome cycle. Bradfield and Errara (1949) observed that -SH reactants prevent the decrease of absorption on continuous irradiation at 2,650 Å and interpret this as an inhibition of the depolymerizing effect of ultra-violet light; the present observations may have some relation with the observations of these authors. Cytochemical methods are not yet sufficiently developed adequately to map out the distribution of -SH groups within the cell, but it is known that both in the nucleus and in the cytoplasm are nucleoproteins comparatively rich in sulphur.

One of the enzymes shown by Brachet and Jenner (1944) to be present in isolated mitochondria is cathepsin, a sulphydryl enzyme. Recently Maver and Greco (1949) have shown that a ribonuclease and a desoxy-ribonuclease are associated with cathepsin prepared from spleen and thymus, and that these two nucleases differ in several ways from the corresponding pancreatic enzymes. These observations are of great interest from our present point of view, for neither of the pancreatic enzymes are inhibited by -SH reactants. Marshak (1948) has already produced evidence that nuclear ribonuclease differs from the pancreatic enzyme. Cathepsin has been considered to be inactive within the living cell, because of its low pH optimum, but the argument is equally relevant to the acid phosphatases of animal tissues, to which it is usually not applied.

However, even if the nucleases which operate during mitosis have any relation to those which Maver and Greco have described, we are still left with the fact, already briefly discussed by Hughes (1949), that nuclear reconstruction is so much more sensitive than prophase to both iodoacetamide and chloracetophenone. Possibly it is not the same enzyme which both dissolves the nucleoli at the end of prophase, and reforms them in telophase; or again, in the young daughter cells, nuclear reconstruction may be linked to synthetic reactions, which are sensitive to sulphydryl reactants.

Although the task of relating the events of cell division to the operation of particular enzyme systems has hardly yet begun, nevertheless it is possible to characterize certain sensitive points in mitosis by their varying susceptibility to a number of inhibitors. On this basis, suggestions have arisen for further lines of research.

REFERENCES

ALBAUM, H. G., NOVIKOFF, A. B., and OGAR, M., 1946. J. biol. Chem., **165**, 125.
—— TEPPERMAN, J., and BODANSKY, O., 1946. Ibid., **164**, 45.
ALLSOP, C. B., and FELL, H. B., 1950. In the press.
BACQ, Z. M., GOFFART, M., and ANGENOT, P., 1940. Bull. Acad. Roy. Med. Belg., **5**, 225.
BARRON, E. S. G., 1943. *Advances in Enzymology*, vol. 3, p. 157. New York (Interscience).
BASTRUP-MADSEN, P., 1949. Acta Path. et Microbiol. Scand., **26**, 93.
BEAMS, H. W., and EVANS, T. C., 1939. Biol. Bull., **77**, 328.
BEATTY, R. A., 1949. Nature, **163**, 644.
BOREI, H., 1945. Archiv. Kemi Mineral. Geol., **20A**, No. 8.
BRACHET, J., and JENNER, R., 1944. Enzymologia, **11**, 196.
BRADFIELD, J. R. G., and ERRARA, M., 1949. Nature, **164**, 532.
BULLOUGH, W. S., 1949. J. exp. Biol., **26**, 83.
BURROWS, M. T., 1921. Proc. Soc. exp. Biol. Med., **18**, 133.

Buschke, W., Friedenwald, J. S., and Fleischmann, W., 1943. Bull. J. Hopk. Hosp., **73,** 143.

Cosulich, D. B., and Smith, J. M., 1948. J. Amer. Chem. Soc., **70,** 1922.

Darlington, C. O., and Koller, P. C., 1947. Heredity, **1,** 187.

Davson, H., 1941, J. cell. comp. Physiol., **18,** 173.

—— and Danielli, J. F., 1938. Biochem. J., **32,** 991.

Dixon, M., and Needham, D. M., 1946. Nature, **158,** 432.

Dustin, P., 1947. Ibid., **159,** 794.

Ewig, W., 1929. Khn. Wschr., **8,** 830.

Fell, H. B., and Hughes, A. F., 1949. Quart. J. micr. Sci., **90,** 37.

Gaulden, M. E., and Carlson, J. G., 1947. Genetics, **32,** 87.

Hughes, A. F., 1949. J. R. micr. Soc., **69,** 215.

—— and Fell, H. B., 1949. Quart. J. micr. Sci., **90,** 37.

—— and Swann, M. M., 1948. J. exp. Biol., **25,** 45.

Kalnitsky, G., 1948. Arch. Biochem., **17,** 403.

Kisch, B., 1934. Biochem. Z., **273,** 345.

Lasnitzski, I., 1949. In the press.

Levan, A., and Östergren, G., 1943. Hereditas, **29,** 381.

Lewis, W. H., and Lewis, M. R., 1924. In Cowdry, *General Cytology*, p. 412. Chicago (University Press).

Liebecq, C., and Peters, R. A., 1949. Biochem. Biophys. Acta, **3,** 215.

Loveless, A., and Revell, S., 1949. Nature, **164,** 938.

Ludford, R. J., 1936. Arch. exp. Zellforsch., **18,** 411.

McElroy, W. D., 1947. Quart. Rev. Biol., **22,** 25.

Marshak, A., 1948. J. cell. comp. Physiol., **32,** 381.

Martin, G. J., and Beiler, J. M., 1947. Arch. Biochem., **15,** 201.

Maver, M. E., and Greco, A. E., 1949. J. biol. Chem., **181,** 861.

Meyer, L. M., 1948. Trans. N.Y. Acad. Sci., **10,** 99.

Miszurski, B., 1949. Exp. Cell. Research. Supp. 1. 450. (Proc. 6th Int. Congress. exp. Cytol. Stockholm.)

Moore, A. E., Stock, C. C., Suguira, K., and Rhoads, C. P., 1949. Proc. Soc. exp. Biol. Med., **70,** 396.

Novikoff, A. B., Potter, V. R., and Le Page, G. A., 1948. J. biol. Chem., **173,** 239.

Östergren, G., 1944. Hereditas, **30,** 426.

—— and Levan, A., 1943. Ibid., **29,** 496.

Pincus, G., and Waddington, C. H., 1939. J. Hered., **30,** 514.

Pomerat, C. M., and Willmer, E. N., 1939. J. exp. Biol., **16,** 232.

Rothstein, A., and Meier, R., 1948. Fed. Proc., **7,** 252.

Schoenbach, E. B., 1949. Cancer, **2,** 57.

Seeger, D. R., Smith, J. M., and Hultquist, M. E., 1947. J. Amer. Chem. Soc., **69,** 2567.

Sexton, W. A., 1949. *Chemical Constitution and Biological Activity.* London (Spon).

Stickland, L. H., 1941. Biochem. J., **35,** 859.

Stokstad, E. L., Regan, M., Franklin, A. L., and Jukes, T. H., 1948. Fed. Proc., **7,** 193.

Trim, A. R., and Alexander, A. E., 1949. Symp. Soc. exp. Biol. III. 111. (Cambridge Univ. Press.)

Verne, J., and Vilter, V., 1940. C. R. Soc. biol., **133,** 621.

Wagley, P. F., and Morgan, H. R., 1948. Bull. J. Hopk. Hosp., **83,** 275.

Warburg, O., and Christian, W., 1941. Naturwiss., **29,** 589.

Willbrandt, W., 1938. Trans. Farad. Soc., **33,** 956.

EXPLANATION OF PLATES

Plates I to IV are photomicrographs of cells in cultures of chick bone which were filmed by phase contrast during the course of an experiment, and then photographed after fixation in Maximov's fluid and staining in Erhlich's haematoxylin. In Figs. 10 16 the phase contrast pictures during life are omitted. Magnification × 2,000. The times given are those between the application of the agent and photography. A key to Plates I IV is given on p. 278.

PLATE I

FIG. 1. Effect of 0·0027 mMolar colchicine on a cell in prophase.
- (*a*) 2 min. Nucleoli still present.
- (*b*) 6½ min. Nucleoli have just disappeared.
- (*c*) Fixation after 39 min. The chromosomes are tightly clumped.

FIG. 2. Effect of 0·57 mMolar aminopterin on a cell in prophase.
- (*a*) 7 min. Nucleoli have just disappeared.
- (*b*) 9 min. The chromosomes are contracting.
- (*c*) Fixation after 37 min. The chromosomes are tightly clumped.

FIG. 3. Effect of 0·65 mMolar chloracetophenone on a cell in prophase.
- (*a*) 1 min. Nucleoli still present.
- (*b*) 5 min. Nucleoli have just disappeared.
- (*c*) Fixation after 17 min.

PLATE II

FIG. 4. Effect of 0·45 mMolar aminopterin on a cell in metaphase.
- (*a*) ½ min. Chromosomes in normal metaphase position and movement.
- (*b*) 8 min. Chromosomes clumping together, no longer in movement.
- (*c*) Fixation after 20 min. Metaphase reconstruction has begun, with a nuclear membrane round all but three chromosomes.

FIG. 5. Effect of 0·075 mMolar Amino-N-mustard on a cell in metaphase. Culture treated with agent for 15 min.
- (*a*) 107 min. Spindle inhibition.
- (*b*) 127 min. Metaphase reconstruction has begun.
- (*c*) Fixation after 183 min. A large nucleus with nucleoli, chromocentres, and chromonemata has formed.

FIGS. 6 and 7. Effect of 0·45 mMolar aminopterin on two cells in early anaphase.

FIG. 6. (*a*) 3 min.
- (*b*) Fixation after 16 min. Arrest of anaphase movement. No cleavage or reconstruction (Text-fig. 1A).

FIG. 7. (*a*) 3 min.
- (*b*) Fixation after 19 min. Arrest of anaphase movement. No cleavage but reconstruction of daughter chromosome groups (Text-fig. 1B).

PLATE III

FIG. 8. Effect of 15 mMolar NaF on a cell in prophase.
- (*a*) 27 min. Nucleoli have just disappeared.
- (*b*) 32½ min. Chromosomes fully formed, but not yet in metaphase position.
- (*c*) 63½ min. Cell in metaphase. Normal random movement of chromosomes just beginning.
- (*d*) Fixation after 59 min. Cell still in apparently normal metaphase.

FIG. 9. Effect of 15 mMolar NaF on a cell in metaphase.
- (*a*) 13 min. Normal metaphase.
- (*b*) 34 min. Early anaphase.
- (*c*) 51 min. Delayed cleavage just beginning.
- (*d*) Fixation after 66 min. Nuclear reconstruction greatly retarded.

FIG. 10. Effect of 10 mMolar NaCN added to a cell in metaphase. Anaphase after 6 min. Fixation after 52 min. Reconstruction of daughter nuclei greatly retarded.

FIG. 11. Effect of 10 mMolar $CH_2FCOONa$ added to a cell in prophase. Normal mitosis; anaphase after 25 min. Fixation after 53 min. Normal telophase and reconstruction of daughter nuclei. Compare with Pl. IV, Fig. 13.

PLATE IV

FIG. 12. Effect of 0·037 Amino-N-mustard on a cell in mitosis. Culture treated with the agent for 15 min. Cell in anaphase 88 min. afterwards. Fixation after 117 min. Normal telophase and reconstruction of daughter nuclei. Compare with Fig. 13.

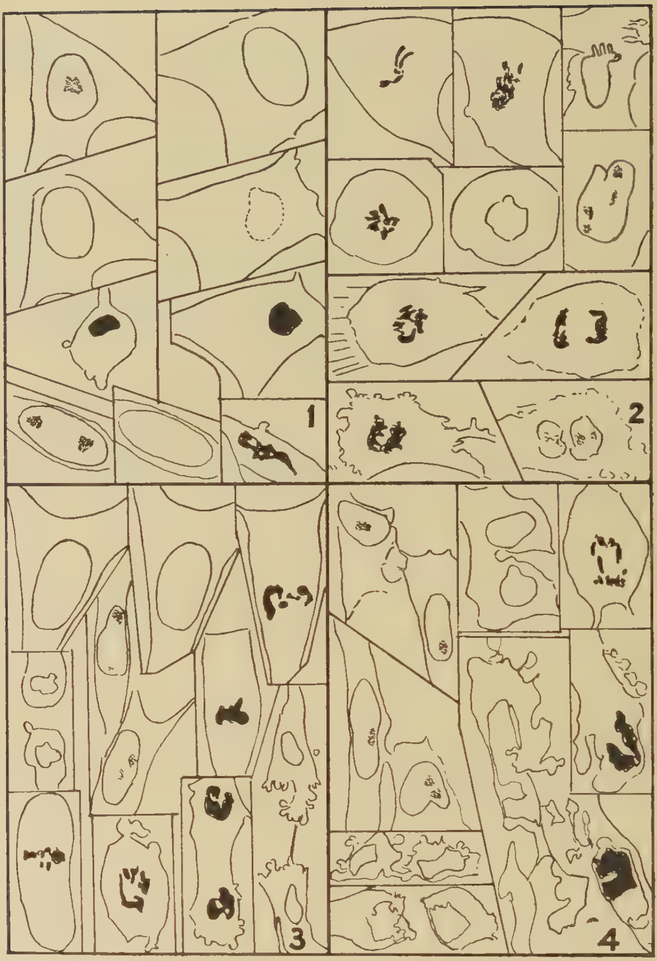
FIG. 13. A cell in telophase in a control culture, treated with Tyrode. Anaphase after 4 min. Fixation after 28 min. Nucleoli chromocentres and chromonemata are visible in daughter nuclei.

FIG. 14. Effect of 1·0 mMolar Na_2SeO_3 added to a cell in metaphase. Anaphase after 7 min. Fixation after 42 min. Reconstruction retarded in daughter nuclei.

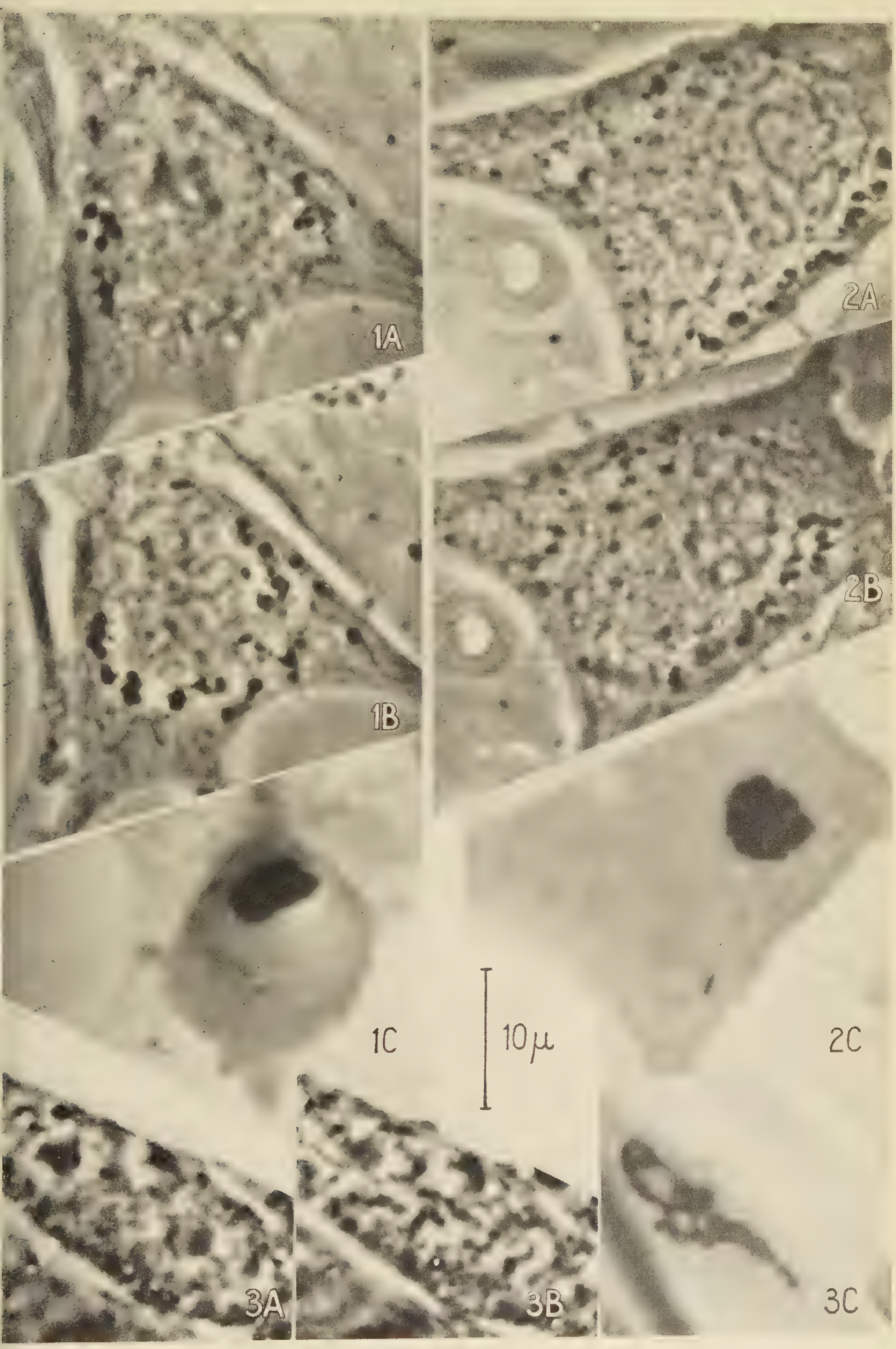
FIG. 15. Effect of 0·45 mMolar aminopterin added to a cell in metaphase. Anaphase after 2 min. Fixation after 34 min. Reconstruction retarded in daughter nuclei.

FIG. 16. Effect of 5·4 mMolar iodoacetate added to a cell as anaphase began. Fixation after 38 min. Reconstruction retarded in daughter nuclei.

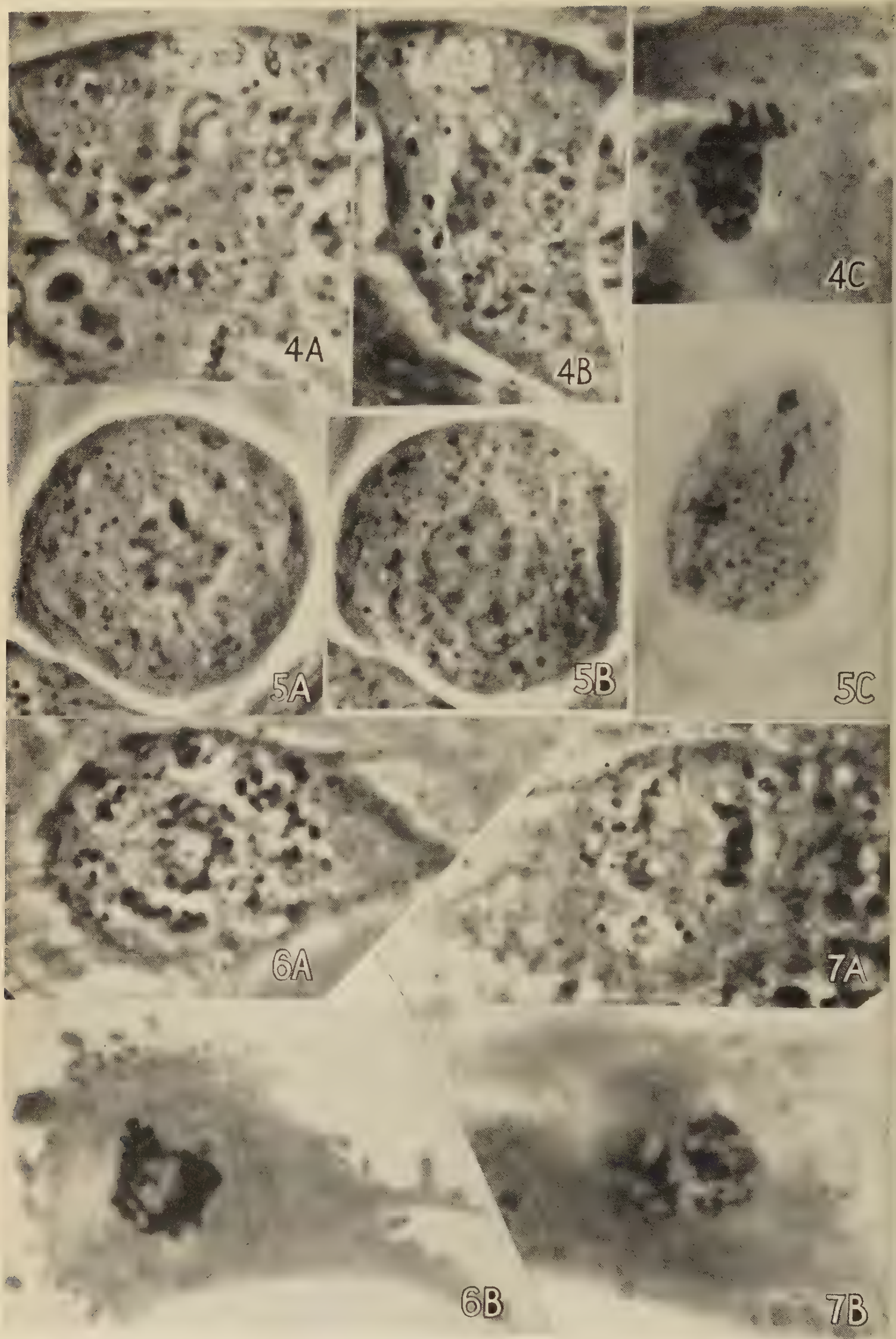
FIG. 17. Effect of 0·13 mMolar chloracetophenone added as anaphase began.
- (*a*) Within $\frac{1}{2}$ min.
- (*b*) 6 min. The daughter chromosomes are together at one end of the cell.
- (*c*) Fixation after 24 min. The cell now consists only of strands connecting blebs of protoplasm, in one of which are the unchanged daughter chromosome groups.



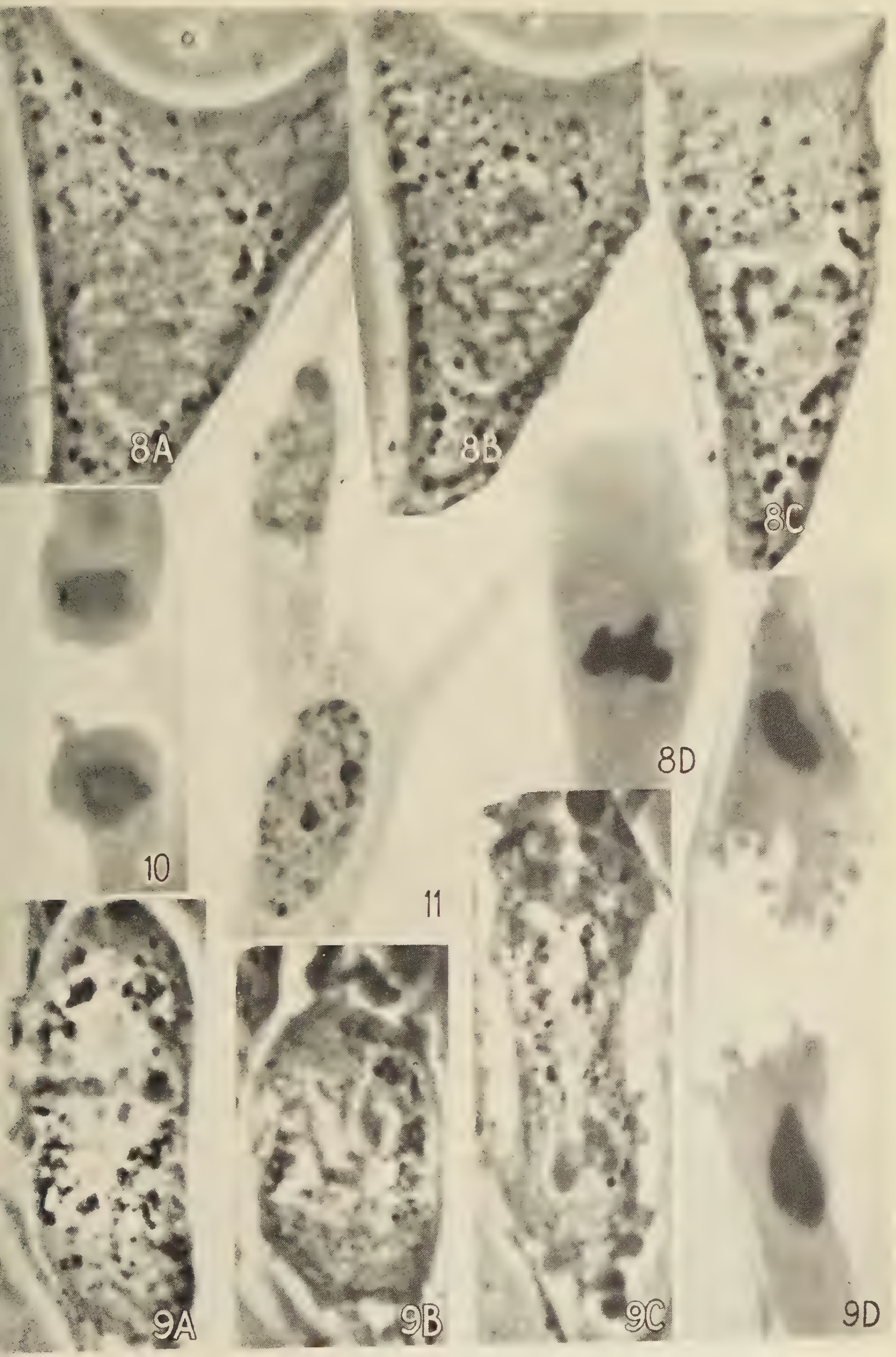
Key to Plates I–IV. Cell margins and nuclear membranes are outlined, nucleoli are dotted, and chromosomes and groups of chromosomes are black.



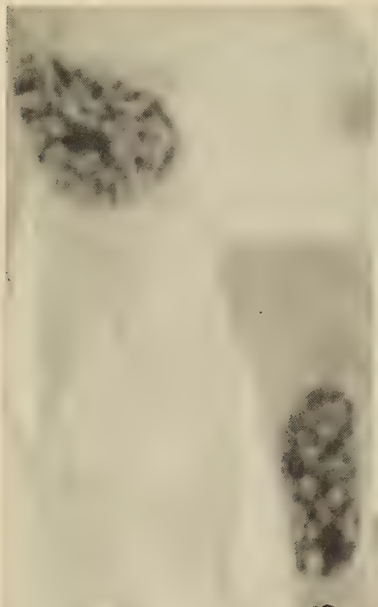
A. F. W. HUGHES—PLATE I



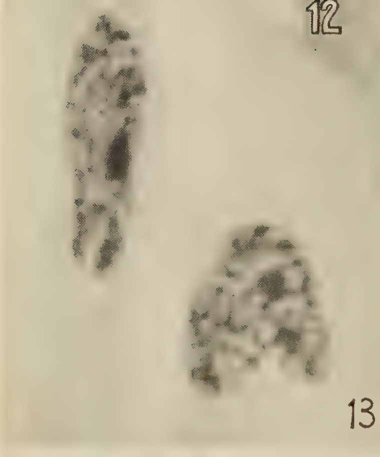
A. F. W. HUGHES—PLATE II



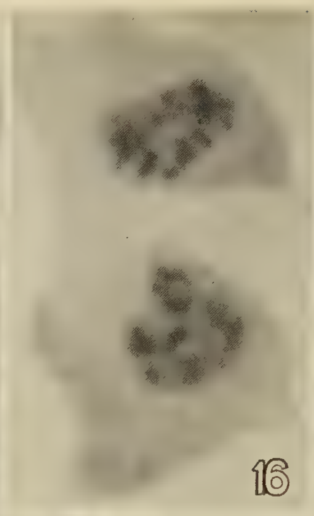
A. F. W. HUGHES—PLATE III



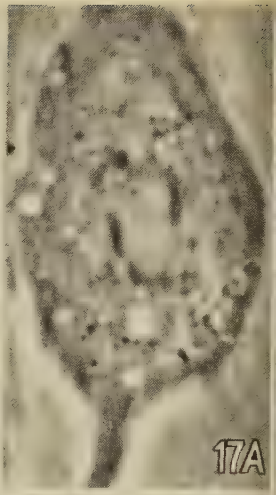
12



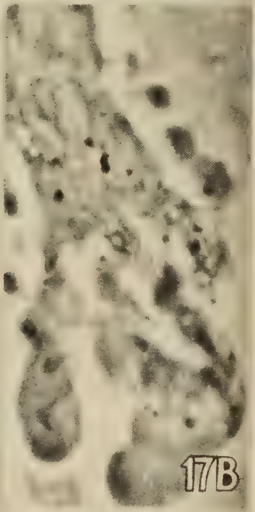
13



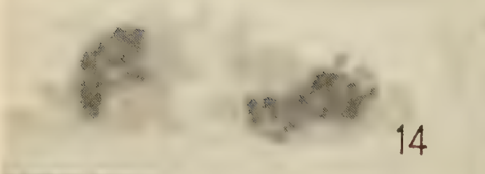
16



17A

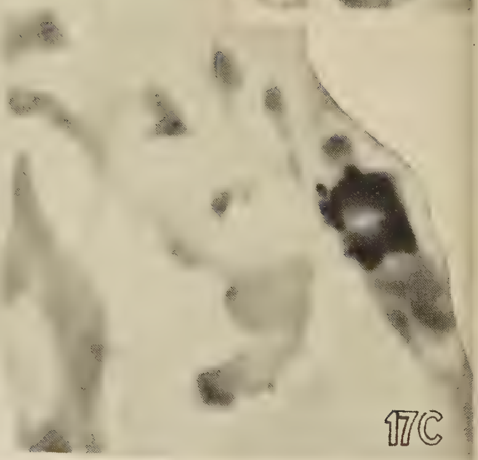


17B



14

15



17C

A. F. W. HUGHES—PLATE IV

Investigations of the Biology of *Peranema trichophorum* (Euglenineae)

By Y. T. CHEN

(*University of Peking; from the Botany School, Cambridge, England*)

SUMMARY

The feeding apparatus of *Peranema trichophorum*, consisting of cytostome and rod-organ, is independent of the reservoir system; the latter is the same in structure and function as that of other Euglenineae. There are two flagella, one directed forward, the other backward and adherent to the ventral body surface. The anterior flagellum is longer and thicker than the adherent one. Both flagella are composed of a central core and an outer sheath. Electron micrographs suggest that the *core* consists of many longitudinal fibrils, and the *sheath* of many short fibrils radiating from the core, giving the whole flagellum the appearance of a test-tube brush. Treatment with certain protein-dispersing agents cause the unfixed anterior flagellum to dissociate into three fibrils.

Peranema multiplies freely on a diet of living yeast-cells; dead yeast is not suitable. *Euglena viridis*, *E. gracilis*, and certain other unicellular algae can also serve as food. Egg-yolk, and especially milk, can be used to maintain bacteria-free pure cultures. Casein is suitable in combination with soil-extract or beef-extract, but never as good as milk. With the latter the individuals are larger and more numerous than with yeast as food, although the cultures decline earlier. Clear liquid media of many various kinds did not support growth: particulate food seems to be essential.

Peranema is capable of ingesting a great variety of living organisms provided these are motionless. Small organisms are swallowed whole; larger ones are either engulfed or cut open by the rod-organ and their contents sucked out. The rod-organ can be protruded out of the cytostome and used in holding on to, and cutting, the periplast of the prey. Starch-grains, oil-droplets, and protein-particles are engulfed and digested. The main food reserves are paramylon-granules and oil-droplets.

H^+ ions, decomposition products of proteins, and other substances diffusing out of living, and particularly dead, organisms attract gliding *Peranema*. Chemotaxis plays an important role in leading *Peranema* to its prey.

Both gliding and swimming have been observed in normal individuals, although the latter is less frequent. While there can be no doubt that swimming results from the action of the anterior flagellum, it does not seem to play an appreciable part in gliding. Nothing is known about the function of the adherent flagellum.

CONTENTS

PAGE

INTRODUCTION 280
MORPHOLOGY 281
CULTIVATION AND GROWTH 285
  1. Preliminary Experiments 285
  2. Bacteria-free Cultures 288
FEEDING HABITS 290
DIGESTION 296
MOVEMENT AND CHEMOTAXIS 300
  Movement 300
  Chemotaxis 303
REFERENCES 306

[Quarterly Journal of Microscopical Science, Vol. 91, part 3, September 1950.]

INTRODUCTION

PERANEMA trichophorum (Ehrbg.) Stein, a colourless member of the Euglenineae, is one of the largest zootrophic flagellates and often used for demonstration on account of its conspicuous flagellum. It was, therefore, deemed useful to find reliable methods of maintaining it in the laboratory, since previous attempts at cultivation appeared to rely too much on chance or gave rise to too complex conditions. It was hoped, moreover, that *Peranema* would be found suitable for studying the zootrophic habit of nutrition, of which not much is known in flagellates. Bacteria-free cultures of *Peranema* have not previously been obtained, and the nutritional requirements of the organism were practically unknown.

The structure and function of the rod-organ and the reservoir system have long been matters of controversy. Most of the earlier writers (Stein, 1878; Bütschli, 1876, 1883–7; Saville Kent, 1880–2) believed that the rod-organ functions as a buccal tube through which food passes. This was denied by Klebs (1883, 1893) who accurately described its mode of action and recognized its function in cutting and piercing the pellicle of the prey.

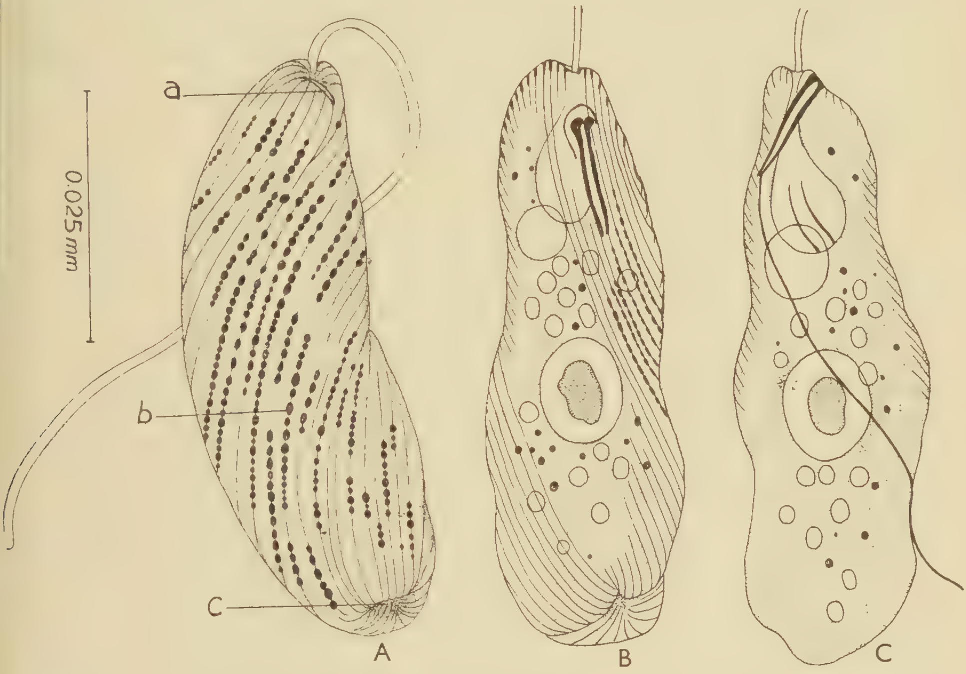
While the structure of the food-ingesting apparatus of *Peranema* was not understood by earlier writers, two different opinions have appeared in more recent publications. It is widely believed that the reservoir system, although of much the same structure as in phototrophic Euglenineae, is used in this organism for ingesting food particles, and that the rod-organ, which is attached to the reservoir, functions mainly as a mechanical support (Jollos, 1925; Hall and Powell, 1927, 1928; Hall, 1933; Lackey, 1933; see also Schaeffer, 1918, for *Jenningsia*, and Loefer, 1931, for *Heteronema*). The other opinion is that, in addition to the reservoir system (which has nothing to do with ingestion), there is another opening, connected with the rod-organ, and used for feeding (Brown, 1930, Pitelka, 1945; see also Rhodes, 1926, for *Heteronema*). Hyman (1936), although accepting the conception that the reservoir is used in feeding, confusedly described an independent rod-organ used in 'strengthening the food-ingesting passage'. It seemed necessary to observe the feeding habits of living individuals in order to clarify the position.

The movements of *Peranema* have been studied by Mast (1912), Lowndes (1936, 1941, 1944, &c.), and others. Lowndes, making use of high-speed cinematography, confirmed Gray's suggestion (1931) that the flagellum is an active unit and is capable of generating at least a part of its own energy. The relation between body movement and flagellar activity, the function of the flagellum, and the chemotaxis of this organism have not hitherto received attention.

Peranema was collected from stagnant waters containing plant residues. Thriving impure and pure cultures in various media have been obtained. With this material the morphology, growth, feeding habits, movements, and chemotaxis have been investigated in the hope of presenting a more complete picture of a common but surprisingly little-known species and contributing to a better understanding of the relationship between coloured and colourless Euglenineae.

MORPHOLOGY

The body of *Peranema trichophorum* is irregularly sack-shaped, with its anterior end tapering and its posterior end rounded, truncated, or sometimes (especially when the organism is poorly nourished) prolonged on one side into a pointed, tail-like process mistaken by James-Clark (1868) for a rudimentary trailing flagellum and used by Skuja (1948) as a specific character.



TEXT-FIG. 1. *Peranema* (diagrammatic). A, B. As seen from the side, showing striation of periplast, cytostome (*a*), protrichocysts (*b*), reservoir, rod-organ, nucleus, contractile vacuole, paramylon grains, area of defecation (*c*). C, The same, as viewed from ventral surface, showing the adherent flagellum.

The dimensions of the body vary with the environmental conditions and have been differently given by previous workers. Newly collected individuals are usually small, with an average size of $42 \times 16\mu$. Under favourable cultural conditions, e.g. in cultures with milk as food, the size increases to $65 \times 25\mu$ on the average, and the individuals are visible to the naked eye as small white specks. In cultures with the yeast *Saccharomyces exiguus* as food the average size is $52 \times 17\mu$.

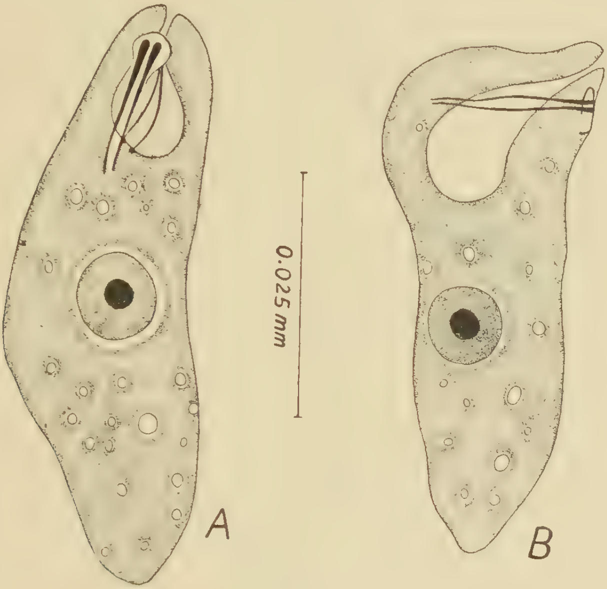
The periplast is finely striated (Text-fig. 1, A, B). The striations are only slightly oblique. Cross-striations have not been seen. Iodine treatment, silver impregnation, night-blue staining, and dark ground illumination render the striae more visible. Running along the striae there are many elliptical or rod-shaped granules, visible in living cells under dark ground illumination or after vital staining (Klebs, 1883; chondriome of Hall, 1926; protrichocysts of Chadefaud, 1938; corps mucifères of Hollande, 1942). In silver-impregnated preparations the striations are seen to converge near the posterior end of the body as they approach a slit-like unimpregnated region, probably the area of defaecation (Hollande, 1942).

Scattered in the cytoplasm are many paramylon granules. They are disk-shaped with a thick rim. When observed from the side they are ovoid. The number and size of these granules vary considerably: they are fewer and smaller (with a diameter of about $1{\cdot}5\,\mu$) in individuals fed with *S. exiguus*, more numerous and larger (diameter about $3\,\mu$) in individuals fed with starch, casein, or milk.

The reservoir, situated in the anterior quarter of the body, is connected by a canal with an opening lying slightly to the right of the anterior end. It divides the latter, as seen in longitudinal optical sections, into a larger and a smaller lip (Text-fig. 1). Posterior to the reservoir is a simple contractile vacuole, the pulsation of which can easily be observed.

The rod-organ is adjacent to the reservoir. It is composed of two parallel rods each of which is hook-shaped with a thickened and slightly curved anterior end and a tapering posterior end (Text-fig. 1). According to Chadefaud (1938) the rods are semicircular in cross-section, i.e. with a furrow on the inner side. They have been mistaken by many workers for the wall of a canal, called the siphon. In agreement with Brown (1930) and Hyman (1936) it was found that the rod-organ is not connected with any part of the reservoir system. The anterior end of the rod-organ is attached to a hyaline, roughly semicircular zone, the position of which is lateral and ventral to, but not connected with, the reservoir canal (Text-fig. 1B). As this zone usually overlaps the reservoir canal, the attached rod-organ also appears to be connected with the reservoir. However, normally gliding individuals have repeatedly been observed with the hyaline zone and the rod-organ completely separated from any part of the reservoir system (Text-fig. 1, B, C). A lateral view in fixed and stained specimens shows the rod-organ again to be ventral to, but independent of, the reservoir. The clear zone in front of the rod-organ is regarded by Brown (1930) and Pitelka (1945), but not by most other authors, as the true ingestive opening. Hyman (1936) has seen this structure too but does not describe it as an opening. The results of my feeding experiments (p. 292) are in favour of Brown's and Pitelka's conclusion, and the term cytostome will therefore be used to denote this opening. The cytostome has a thickened anterior margin described by Brown (1930), Chadefaud (1938), and Hollande (1942) as a third, the falcate rod. As it is not connected with the two parallel rods and does act together with them during feeding, it is perhaps better regarded as a stiffened rim of the cytostome than as part of the rod-organ (Text-fig. 2, A, B).

Hall and Powell (1928) and Hall (1934) deny the existence of a second opening, but some of their figures (1928, pl. I, 1, 2, 3, 4) indicate the presence of two openings in the same individual. In their figures an opening at the anterior end of the body is connected by a canal to a second opening, labelled 'cytostome'. I also saw two openings but found that at the anterior end to lead into the reservoir from which the flagella protrude, while the other (posterior and ventral to the reservoir opening) is the true cytostome. In the figures of Lackey (1933) the cytostome is connected with the part of the reservoir canal in front of it, thus giving the appearance of a 'tuning-fork shaped cytostome'.



TEXT-FIG. 2. *Peranema*, Schaudinn/Heidenhain. A, viewed from ventral surface. B, twisted cell, viewed from the side.

Neither in living nor in stained specimens could a canal leading from the cytostome into the body be traced. It seems that there is not a permanent gullet, and that food enters through a temporary passage.

From the oblique position of the rod-organ it appears that bilateral symmetry, so often given as a diagnostic character of the Peranemaceae, does not, in fact, exist in *Peranema*. Klebs (1883) seems to be the first who has used the term 'bilateral', but not in the correct sense: he meant 'dorsoventral'. Lemmermann (1913), Doflein (1916), and others followed him without being aware of the inaccuracy. Dorsoventrality is very distinct in *Peranema* which glides only on one surface, the ventral side of the body (cf. p. 301). When individuals glide along the bottom of the container, they are observed through the dorsal surface with the posterior end of the rod-organ pointing towards the right as seen under a compound microscope (Text-fig. 9A, p. 299). When they glide along the upper surface of the water, however, it is the ventral surface of the body which is observed, and the posterior end of the rod-organ points to the left (Text-figs. 1C, 2A).

There are two flagella. The one directed forward is the one known alone to most of the previous authors. It is wider than the second one and up to twice the length of the extended body. The second flagellum is much narrower and only two-thirds the length of the body. It turns back immediately after protruding from the reservoir opening and adheres to the ventral body surface, simulating a streak on the periplast (Korshikoff, 1923; Lackey, 1929, 1933; Hall, 1934). This second flagellum is visible in living specimens under dark ground illumination or under the phase-contrast microscope. By gentle pressure or by treatment with osmium tetroxide it can be detached. Skuja (1948) doubts the existence of the second flagellum and believes that what is considered as a second flagellum may only be a daughter flagellum of a dividing individual. It seems unlikely, however, that a newly grown flagellum should adhere to the periplast or that it should be narrower than the primary flagellum.

In stained specimens the two flagella are seen to originate independently from two basal granules which lie side by side in the wall of the reservoir, as in other Euglenineae. There is no bifurcation of flagellar roots. What Doflein (1916) and Brown (1930) describe as bifurcation seems to be a misinterpretation due to failure to observe the second flagellum. The structure of the flagellar apparatus of *Peranema* therefore accords with the views of Pringsheim (1948) regarding the flagellar apparatus of the Euglenineae.

Save for the difference in size, the anterior and posterior flagella have the same structure. Each is differentiated into an axial core and an outer sheath which can be observed under the light microscope, particularly after treatment with Loeffler's stain, under dark ground illumination, or under the electron microscope. The latter reveals still finer structures (Text-fig. 3A, a). Contrary to Lackey's statement, but in accordance with Hollande (1942), the sheath covers the whole length of the anterior flagellum except for the part within the reservoir (the root) which, when squeezed out by the contraction of the body during the preparation of specimens, is seen to be naked (Text-fig. 3B a). The sheath seems to consist of numerous fibrils radiating from the axis, thus giving the whole flagellum the appearance of a test-tube brush. It is in this respect different from the flagellum of *Euglena* where the sheath consists of longer fibrils wound spirally round the axis (Brown, 1945, and own electron-micrographs).

The axial core shows in gold-shadowed specimens longitudinal striation, indicating fibrillar differentiation. The number of fibrils, as revealed by the electron-microscope, is considerably higher than the two of Brown (1945, 1946) or the four of Dellinger (1909). The same has been found in several other Euglenineae, including *Euglena gracilis* (Brown's material). My conclusions are in agreement with Korshikoff's (1923) and those of Schmitt, Hall, and Jakus (1943) that the axis consists of a relatively large number of thin fibrillae. The two thick fibrils, found by Brown in *Euglena gracilis* and

'*Astasia Klebsii*' (in fact the colourless race of *E. gracilis*), may be bundles of fibrillae situated in the edges of the ribbon-shaped flagella, as suggested by my electron-micrographs.

By the application of protein-dispersing reagents an attempt was made to render the inner structure of the flagellum better recognizable. With previously fixed material no results were obtained, but the treatment of unfixed individuals with chemicals, such as urea, known to disperse fibrillar proteins stabilized by electro-valencies, constantly leads to the separation of the anterior flagellum into three fibrils, possibly as a result of the dissolution of the sheath. While in some of these reagents, e.g. urea (from saturated to 3 M solution), NaOH (0·1 0·2 N), &c., the fibrils ultimately dissolve, in Na_2SO_3 (2–8 per cent.), dissolution is only partial even after 24 hours. In HCl (0·2 -5 N), on the other hand, the flagellum remains intact, even after treatment for 24 hours.

Like Deflandre (1934*a*, 1934*b*), who used nigrosin, and Hollande (1942), I could not find in *Peranema* the lash-like processes which, according to Vlk (1938), should be present on the surface of the flagella of all members of the Euglenineae. Neither Loeffler's mordant staining technique, nor electron-microphotographs showed any trace of such appendices, although both methods were successful in various species of *Euglena*, *Trachelomonas*, and *Phacus* (Pringsheim and Hovasse, 1950).

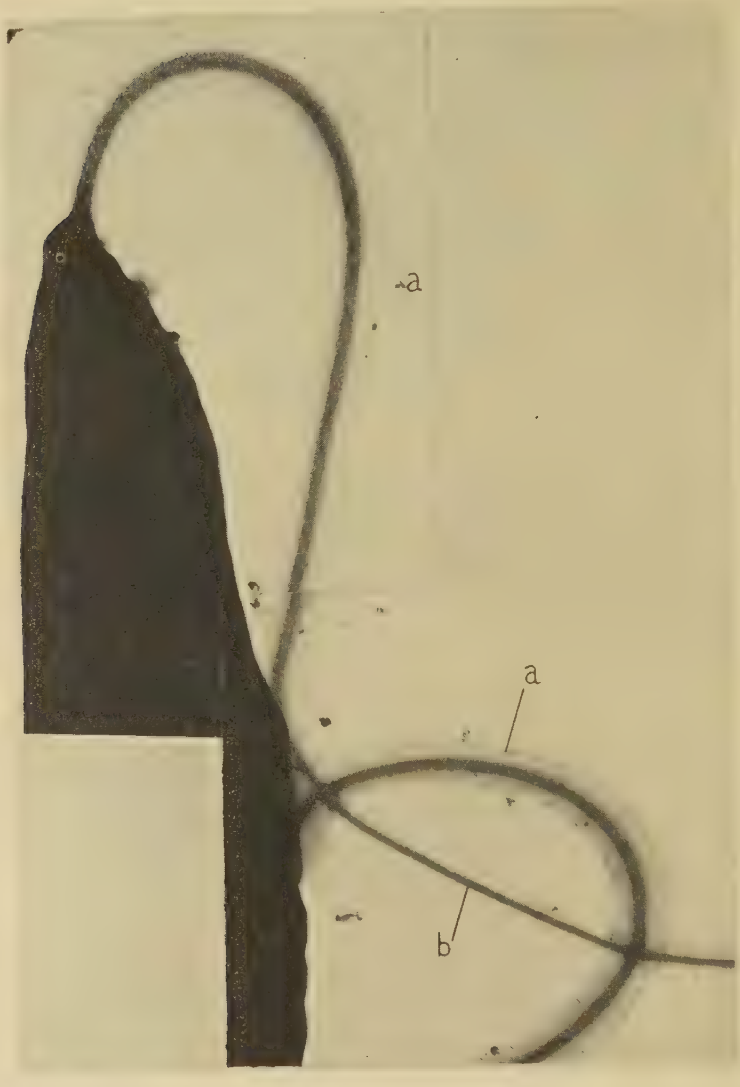
CULTIVATION AND GROWTH

Peranema was cultivated by Lackey (1927) in a wheat-water medium and by Hall and Powell (1927, 1928) and Mast and Hawk (1938) in a beef-suet infusion, all with bacteria as food. Brown (1930) maintained that a culture of *Peranema* will not live unless inoculated with some euglenoid flagellate, but Shettles (1937) used a balanced salt solution with rice grains and *Chilomonas* as food.

1. *Preliminary experiments*

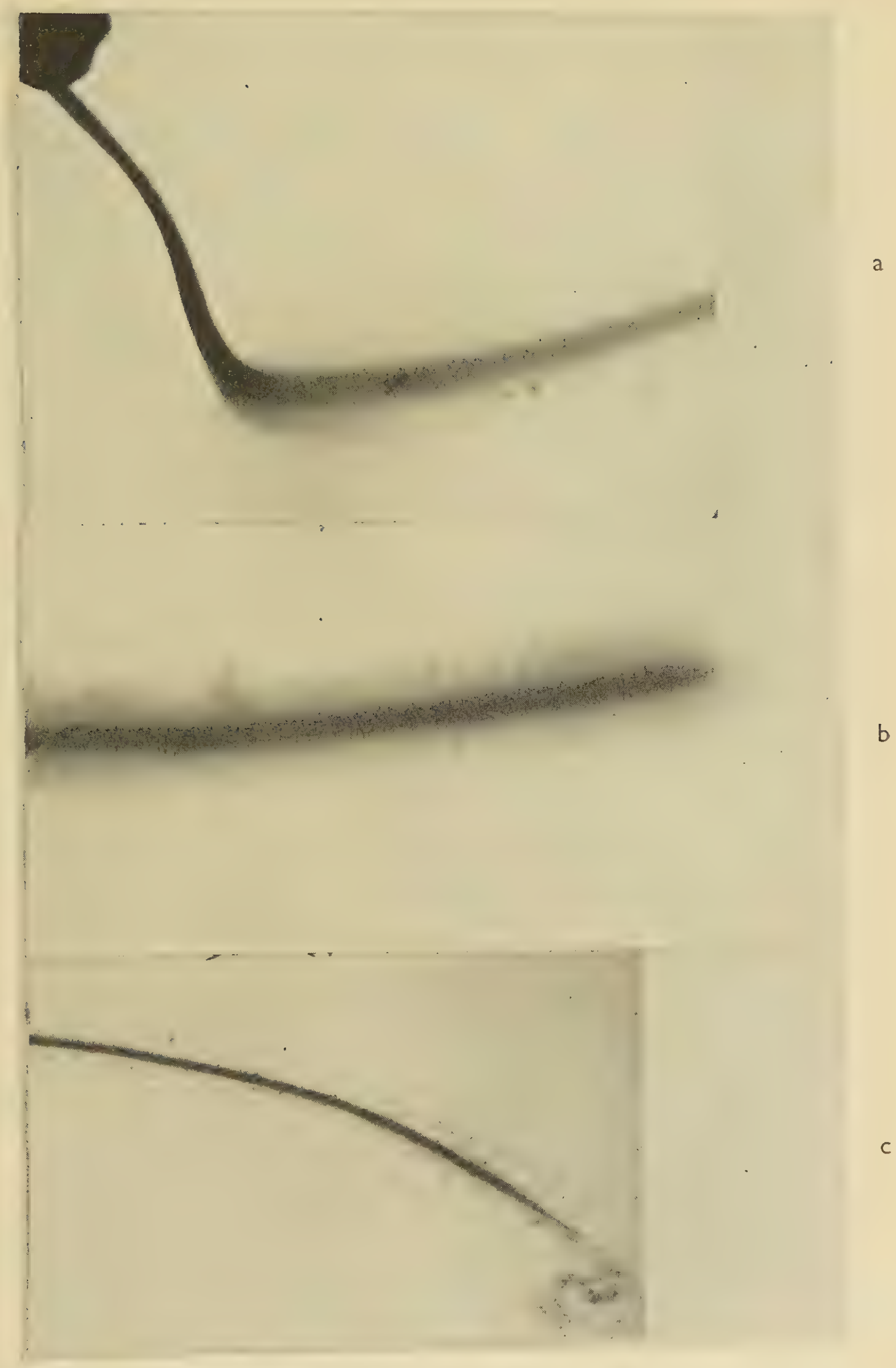
The material with which I started had been grown for some time with *Saccharomyces exiguus* as food suspended in half-saturated calcium sulphate solution, a method which had previously been devised for *Paramecium* (Pringsheim, 1928). Cultures were fed daily during the first week, and less frequently later on. Flourishing cultures were obtained after about a month. Wheat and hay infusions and various bacteria from agar slopes did not support satisfactory growth.

With *Saccharomyces exiguus* as food the following solutions, apart from calcium sulphate, were tried: half-saturated aqueous calcium carbonate; diluted Benecke solution; one-tenth diluted natural and artificial sea-water; soil extract and glass-distilled water. *Peranema* multiplies in all these solutions: least well in distilled water, best in soil extract and almost as well in Benecke solution, which both accelerate the rate of multiplication compared with calcium sulphate solution, although they do not give a denser peak population.



TEXT-FIG. 3A. Electron-micrographs of the flagella of *Peranema*. *a*, curved anterior flagellum; *b*, posterior flagellum; osmium tetroxide. × 12,000.

That *Peranema* can grow in distilled water does not imply that it needs no mineral salts, since yeast present in the culture supplies a certain amount. This is borne out by the facts that the health of the culture is poor in the beginning but improves after repeated feeding and that without feeding *Peranema* soon dies in distilled water but can live several days in solutions with calcium (cf. Pringsheim, 1928, for *Paramecium*). Accordingly addition of very low concentrations of calcium salts greatly improves the cultures,



TEXT-FIG. 3B. (*a*) root of anterior flagellum squeezed out of reservoir; (*b*) end of anterior flagellum showing core and sheath; (*c*) end of posterior flagellum; sheath much less dense. × 16,000.

one-sixteenth to one-sixty-fourth being better than more concentrated solutions.

Apart from *Saccharomyces exiguus* the following organisms from pure cultures were tested as food: *Polytoma uvella* Ehrenb., *Chlorella pyrenoidosa* Chick, *Prototheca Zopfii* Krüger, *Stichococcus bacillaris* Naeg., *Chilomonas paramecium* Ehrenb., *Euglena gracilis* Klebs, *Astasia longa* Pringsheim, *Saccharomyces cerevisiae* Hansen, and *Rhodotorula glutinis* Fresenius. Only *S. cerevisiae* is as good as *S. exiguus* in maintaining *Peranema. Rhodotorula*, taxonomically near to it, may be inferior because it cannot be suspended in water. *Chlorella* in the light grows so fast that *Peranema* is eventually suppressed. The two species of Euglenineae as well as *Stichococcus* only support moderate growth. Between a green *Euglena* and *Peranema* there is a complicated balance in multiplication, the former first growing quicker, then *Peranema* catching up, until eventually, when the food organism declines, the latter also gradually decreases until after about a year only a few individuals remain.

The influence of the pH has been investigated by using a series of Clark's buffer solutions ranging from pH 4 to 9. With *S. exiguus* as food *Peranema* was found to multiply between 5·2 and 8·4, with an optimum at neutrality. In order to see whether *Peranema* is capable of becoming adapted to higher concentrations of mineral salts, dilutions of artificial sea-water ranging from 1/20 to 1/4 were inoculated with *Peranema*. The growth was normal in the lowest concentration, and there was none in 1/5 and 1/4. If inoculated from 1/20, growth occurred up to 1/5. Even if the increase of concentration was effected by gradual evaporation of a culture in 1/20 sea-water, *Peranema* could tolerate only a concentration of 1/4·2 (24 per cent. sea-water). Compared with other flagellates (Finlay, 1930; Hardin, 1942) *Peranema* has only a very restricted capacity of adapting itself to salt-water.

2. *Bacteria-free cultures*

Peranema purified by washing (Pringsheim, 1946) could be grown with *Saccharomyces* as food in two-member pure cultures. Healthy cultures were, however, only obtained with soil extract as the medium. No growth occurred in dilute sea-water or calcium sulphate solutions, even after repeated inoculation. As both these media support growth when bacteria are present, bacteria evidently play some part in the nutrition of *Peranema*, although they alone do not suffice to maintain the cultures. Butterfield (1929), Lilly (1942), and Hardin (1944) had similar results with various other protozoa. Since soil-extract works well, whether bacteria are present or not, it seems that some substances produced by bacteria and beneficial to the growth of *Peranema* can adequately be supplied by soil extract. Attempts to culture *Peranema* on yeast killed by heat, even as low as 80°, failed.

Really pure and thriving cultures were obtained with cow's milk. One drop of milk was added to 8 c.c. of either distilled water, calcium sulphate solution, or 1/10 sea-water. The resulting mixtures were heated in a steam-chamber for an hour on three consecutive days, and inoculated with single, washed individuals. Multiplication in all three media was so intense that after 3 weeks the inner surface was densly covered with *Peranema*—large, healthy individuals containing numerous food vacuoles. Tests proved the sterility of all the cultures. No difference in the rate of multiplication in the three media has been observed. For maintaining the cultures, however, that with calcium sulphate is preferable to the others because during sterilization the milk is coagulated in this medium, and the fluid is then clear, permitting better observation of the organisms. Otherwise the milk emulsion remains turbid until the suspended milk droplets have been ingested by the *Peranema*.

Milk cultures decline earlier than those with yeast as food, ceasing to flourish after 3 months owing mainly to the accumulation of waste products. The decline could not be prevented by adding more milk. Autoclaved milk gave good results initially but not in successive subcultures. Something essential for *Peranema* seems to be destroyed by excessive heating. The optimum concentration of milk is 0·5–1 per cent. Concentrations higher or lower than these do not support appreciable growth. Bacto peptone, Bacto tryptone, yeast extract, glucose, starch, and almond-oil have no effect when added to milk cultures, whereas 0·1-0·2 per cent. beef-extract accelerates growth, although it does not increase the final density of the population.

With milk as food *Peranema* is not only bigger than with yeast (cf. p. 282) but also contains more and larger paramylon grains and oil-droplets, and it multiplies more quickly. In milk cultures the population reaches its maximum of about 17,000 individuals in 1 c.c. after about 1 month. This density is maintained for about 10 days and then falls steadily to a level of about 12,000 individuals which lasts about 50 days; subsequently the population declines rapidly. Yeast cultures reach only a maximum of about 7,000–8,000 in 1 c.c. but last longer, and extinction does not occur until after 10 months or more, provided food is added repeatedly.

Fairly good results were also obtained with egg-yolk as food, although the density of the cultures was never as high as in milk cultures. Mixtures of casein and butter have been tested in an attempt to analyse the effect of the ingredients of milk. Fat-free casein was kindly supplied by Dr. E. Kodíček (Dunn Nutritional Laboratory, Cambridge). To 10 c.c. of distilled water, calcium sulphate solution, soil-extract or 0·1 per cent. beef-extract was added 0·1 per cent. butter plus 0·02 gm. casein. Culturing and subculturing was possible, particularly in the last two media, but none of the cultures was nearly as good as those with milk. Addition of whey, prepared by clotting fresh milk with commercial rennet, gave but little improvement. Butter or an ether extract of milk, both in 0·1 per cent. beef-extract or yeast-extract, as well as casein in soil-extract, beef-extract, or yeast-extract all support moderate growth. Addition of glucose does not improve the results. The optimum concentrations of butter and casein are 0·36 and 0·2 per cent. respectively. On the whole none of these media is satisfactory. Since butter alone does not support growth it seems that *Peranema* can multiply well only when some proteinic substance is present which is digested inside the body. No evidence of proteolytic enzymes being excreted could be found by placing drops of a dense *Peranema* culture on gelatine slopes under aseptic conditions.

Many attempts to cultivate *Peranema* in monophasic liquid media have been made. Of the 45 mixtures tested—various combinations of beef-extract, yeast-extract, liver-extract, tryptone and a number of other peptones in various degrees of hydrolysis, glucose, alkali caseinate, and sterile filtrates of casein solutions, egg-albumin and blood-serum—none supported growth. So far as these results go, *Peranema* seems to be strictly zootrophic, as it requires particulate protein food.

FEEDING HABITS

While the feeding habits of ciliates and rhizopods have been extensively studied, little is known about those of zootrophic flagellates. *Peranema* is relatively large in size and slow in motion, and ingests readily a great variety of food particles. It seemed therefore suitable for observations on behaviour towards food substances.

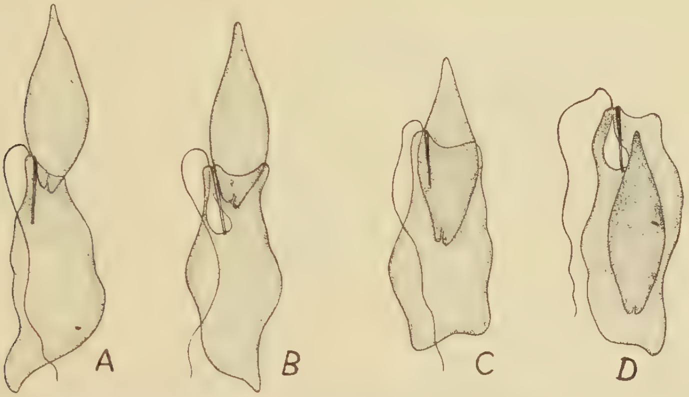
In the first place an answer was sought to the question whether there is any food selection, and experiments were begun in which they were fed: (1) with different kinds of substances separately, to see if there is variation in the frequency of ingestion; (2) with a mixed diet, to see if *Peranema* has the ability to select one and reject the other component; and (3) with non-nutritive coloured substances, continuously for a longer period, to see if *Peranema* can reject these.

A few drops of a *Peranema* culture, containing about 4,000 individuals, were transferred to watch-glasses with a suspension of food-particles or of organisms. At intervals samples were counted in order to determine the ratio of the number of individuals *with*, to that of individuals *without*, the food in question. The result obtained was that, among the substances used, casein was ingested most frequently, while sand-grains and calcium carbonate powder were not taken at all. Between these two extremes, arranged in order from higher to lower frequency of ingestion, were: carmine, *Chlorella pyrenoidosa*, *Stichococcus bacillaris*, *Saccharomyces exiguus*, Chinese ink, *Euglena gracilis*, *E. viridis*, and starch-grains. Thus carmine and Chinese ink, both of no food value, are more frequently ingested than both species of *Euglena*, on which *Peranema* can live.

Carmine was ingested also in the presence of a food substance, for instance casein, *Stichococcus*, or *Saccharomyces*. Like carmine, casein was ingested more readily than any of the organisms tested in mixture with it. Carmine particles are ingested even when the individuals have already taken a considerable amount of real food, e.g. *Euglena* or casein. Satiety does not cause *Peranema* to reject carmine, unlike *Stentor* (according to Schaeffer, 1918). Even by prolonging the supply of carmine, *Peranema* cannot be 'trained' to reject it, as Metalnikow believed to have proved in *Paramecium*.

What there remains of food-selection in *Peranema* has nothing to do with the food-value of the substances, but is determined by physical and chemical properties and, in the case of living organisms, by the motility of the prey.

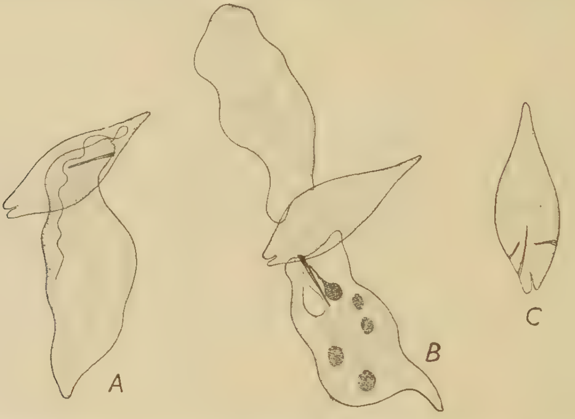
The controversy regarding the mechanism and the place of ingestion has already been mentioned (p. 280). Previous observers had mostly drawn their conclusions from morphological observations (Hall and Powell, 1928; Brown, 1930; Lackey, 1933). It was hoped that feeding experiments would help to clear up both these points. In observing the process of ingestion, hanging-drop preparations were used containing individuals which had been without food for a day. Of the thirteen species used as food Euglenineae of larger size gave the most valuable results. In no case were actively moving forms ingested.



TEXT-FIG. 4. Successive stages (A–D) of *Peranema* swallowing a *Euglena viridis*. The wide extension of the anterior end is seen, and also the independence of the food entrance from the reservoir.

When 10–20 individuals of *Euglena viridis* were added to a hanging drop, some soon became motionless and were then attacked by *Peranema*. The moment the tip of the undulating anterior flagellum of a *Peranema* touched a *Euglena*, the whole flagellum began to beat violently; whereupon the cell-body contracted and the flagellum drew back. Soon the cell relaxed and the flagellum resumed normal movement. The process of contraction and recovery was repeated several times until the body came into contact with the *Euglena*, when ingestion began. It always started at the anterior end of the prey (Text-fig. 4). When a *Peranema* touches the front end of a *Euglena*, the rod-organ together with the adjacent region of the ventral body-surface moves forward from its original position, becoming level with the anterior end, until it touches the *Euglena*. The rod-organ then protrudes a little and becomes attached to the surface of the *Euglena*. The body of the *Peranema* starts to move forward around the prey, while the rod-organ remains attached to the periplast and begins to push the *Euglena* through the widely expanded anterior end of the *Peranema* into the interior. The rod-organ is then detached from the periplast, moves over the surface of the *Euglena* and is re-attached on another part of the periplast, while the body of the *Peranema* follows. By repeatedly attaching, pushing, detaching and re-attaching the rod-organ, with forward movement of the body, the *Euglena* is swallowed. The time required for the whole ingestion varies from 2 to 15 minutes, with an average of 8 minutes. During ingestion the two rods always move together as if connected.

It is not uncommon for a *Peranema* to ingest two *Euglenae* at a time, although its size is scarcely larger than that of the prey. A *Peranema* was seen, 2 minutes after taking in a *Euglena*, to meet another which was then attacked and swallowed immediately. Both of the two ingested *Euglenae* were soon digested. In some instances the second *Euglena* was only partly swallowed and, perhaps because there was no room to spare for it, eventually egested.



Text-fig. 5. *Peranema* cutting open the periplast of *Euglena viridis* and ingesting its protoplasm. A, *Peranema* protrudes into the cell of *Euglena* after perforating its periplast with its rod-organ. B, Two individuals attack the same *Euglena*. The protoplasm of the *Euglena* is sucked in by one of the *Peranemae* through a way separate from the reservoir, which remains clear. C, *Euglena*, after being attacked, shows three splits on the body-surface.

Sometimes a *Peranema*, instead of ingesting a *Euglena* as a whole, cuts open the periplast and sucks out the contents (Text-fig. 5). When the cytostome is applied to the attacked *Euglena*, the rod-organ is protruded and rasps up and down over the periplast of the prey. After 6-10 minutes the protoplasm of the *Euglena* with the green chromatophores begins to flow into the body of the *Peranema*, not through the reservoir but through a passage lateral and nearly parallel to it (Text-fig. 5B). This passage can only be seen when food is passing and may, therefore, be only a temporary, rather than a permanent, structure. The reservoir and its canal, during this process of ingestion, remain clear and free from food particles. Ingested food is accumulated at the posterior end of the passage to form digestive vacuoles which are detached apparently by contractions of the body. The shape of these vacuoles is usually that of the food enclosed, i.e. the food appears to be in direct contact with the surrounding cytoplasm.

Sometimes *Peranema* sucks in only part of the contents of a *Euglena*; in other cases it consumes the whole, leaving only the periplast. As the contents of the prey decrease during the sucking, the anterior end of the *Peranema* may penetrate through a hole in the periplast into the interior and continue to suck there (Text-fig. 5A). The rod-organ, protruding slightly into the cytoplasm of the *Euglena*, seems to help in pushing the food into the body. Sometimes the rod-organ moves slowly along the boundary between the cytoplasm and the periplast, separating the one from the other. The remaining empty periplast shows regularly one or more splits where the rod-organ has been applied (Text-fig. 5C).

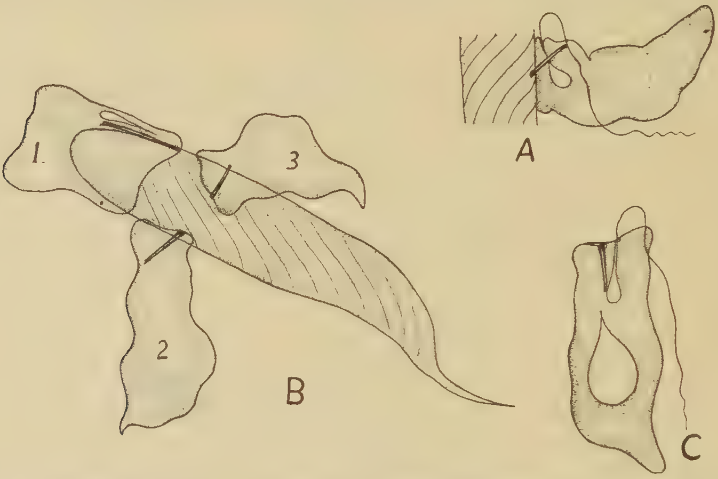
The frequencies of the two types of ingestion, engulfing and sucking, vary with the circumstances. When a *Euglena viridis* is attacked by a single *Peranema*, the whole cell will be swallowed. When it is attacked by two or three, it will be either shared by the predators, each sucking a portion of it, or engulfed by one of them. When it is attacked by many *Peranemae*, it is always pierced and sucked by a number of individuals; swallowing by a single individual has not been observed under such conditions. Towards *Euglena gracilis* and its apochlorotic race ('*Astasia longa*'), *Peranema* behaves in the same way as towards *E. viridis*.

Euglena spirogyra, although much larger than *Peranema* itself, is also attacked, but only when it has been killed, e.g. by pressure or by heat. It is easily killed by pressing the coverslip, and cytoplasm flows out through lesions in the periplast. If then *Peranema* individuals are introduced, they soon attack the wounded *Euglena*. Those which arrive first approach the wounds whence cytoplasm is flowing. Only when such sites are occupied do they begin to cut new openings with the rod-organ. Heat-killed *E. spirogyra* is preferentially attacked at the anterior end of the cell. The front part of *Peranema*, truncated and widely expanded, is applied firmly to the surface of the prey, while the rod-organ moves to cut the periplast. It was observed that, while the cytostome and its attached rod-organ were working in contact with the periplast of the *Euglena*, the anterior end of the *Peranema* bearing the flagellum and the reservoir opening was shifted to one side, i.e. out of contact with the *Euglena* (Text-fig. 6A). The cytoplasm of the prey could be seen flowing through the cytostome into the body while the reservoir remained empty.

When a sucking *Peranema* becomes full of food vacuoles it detaches itself from the *Euglena* and its place is taken by another individual. As the cytoplasm of the prey gradually decreases in volume by continuous sucking, the *Peranema* penetrates more and more deeply into the interior and may come to lie completely within the almost empty body of the prey. In one instance three individuals were seen within the periplast of a single *E. spirogyra*.

If not more than four individuals attack a *E. spirogyra* the one nearest the front end usually tries to engulf it, although this can never be successful owing to the smaller size of *Peranema*. The attack proceeds in the same manner as that on *E. viridis* and, after a few minutes, the anterior end of the *E. spirogyra* sinks into the body of the *Peranema* through its tremendously expanded anterior end (Text-fig. 6B). When the *Peranema* has eaten to capacity, only about one-fifth of the prey is swallowed. As the *Peranema* attempts to engulf more, the rod-organ moves here and there attempting to anchor itself to the periplast, and the body rotates about the axis of the *Euglena*.

It is at this stage that the ingested part of the *Euglena*, sunk deeply into the body of the *Peranema*, is clearly seen to be outside the reservoir, which remains empty and transparent and is pressed to one side of the body (Text-fig. 6B). Eventually the *Euglena* is egested through the cytostome. Immediately after this, while the anterior end of the *Peranema* remains for a short time widely expanded, the independence of the feeding apparatus from the reservoir and its canal is especially clear (Text-fig. 6C). The cytostome, since it has not yet contracted to its normal size, can be observed near the distal end of the rod-organ as a crescent-shaped opening lateral to the reservoir. The space in the body of the *Peranema* previously occupied by the ingested part of the *Euglena* remains as an empty vacuole not connected with the reservoir system. Soon the anterior end is restored to its normal tapering shape, and the cytostome with its attached rods returns to its previous position, ventral and posterior to the reservoir opening, and regains its former appearance as a transparent region near the end of the rod-organ.



TEXT-FIG. 6. *Peranema* feeding on heat-killed *Euglena spirogyra*. A, during the attack; reservoir and flagellum shifted away from the prey; rod-organ seen to be independent of the reservoir. B, Three individuals attacking the same *Euglena*; the one in front is trying to engulf the whole prey, the reservoir remains empty; the other two begin to apply their rod-organs. C, The same individual (1 in B), immediately after egesting the partly swallowed *Euglena*; note the independence of the rod-organ and the cytostome in front of it, from the reservoir; the space, which was previously occupied by the prey, is separated from the reservoir.

Cyclidiopsis acus Korschikoff (=*Astasia linealis* Pringsheim) is much less attractive to *Peranema* than *Euglena spirogyra*, but otherwise affects it in a similar way.

Cannibalism occurs in *Peranema*. When transferred from a culture to a hanging drop, a certain number of individuals are usually killed or injured and shed their flagella. They are soon eaten by the active individuals, their periplasts being perforated and the contents sucked out. No swallowing of the whole organism has been observed.

Chilomonas paramecium is only ingested when motionless, and is then ingested as a whole, without its surface being perforated. Contrary to the statement of Hall (1933) *Chilomonas* was never found to pass through the reservoir system. Hall relied on the observation that in his stained preparations the food vacuole containing the ingested *Chilomonas* appeared to be connected with the reservoir. This, however, does not necessarily mean that the prey is ingested through the reservoir. A food-vacuole with *Chilomonas* occupies a relatively large space, and when the cytoplasm contracts during fixation, the vacuole may seem to be connected with the reservoir.

Cells of *Saccharomyces exiguus* and *S. cerevisiae* are always swallowed whole. For the purpose of observation yeast cells were made more visible by staining with 0·2 per cent. Congo-red solution. When a gliding *Peranema* touches a yeast cell with its flagellum it either contracts and moves away or continues gliding, so that the yeast cell 'flows' along the flagellum to the anterior end of the body. When it arrives there *Peranema* reacts in various ways. It either ingests it or keeps gliding normally, leaving it behind. When ingestion occurs, *Peranema* usually contracts considerably so that, by the time it recovers to its normal shape, the yeast cell is already inside the body. Occasionally, however, the yeast cell is ingested while the *Peranema* maintains normal gliding, without contortion of the body. In the latter case, the passage of the yeast cell into the body can be observed. The observation confirms that it passes from the cytostome into the cytoplasm not through the reservoir system, but through a way near to but not connected with this. Sometimes, however, when the ingested cell lies ventral to the reservoir it seems to be within it, and when it passes posteriorly, it may give the impression that it comes out from the end of the reservoir. A side view will reveal the true position of the yeast cell, ventral to and outside the reservoir. The rod-organ appears to be of little importance in the ingestion of such small organisms. From one to six yeast cells have been observed in a single food-vacuole.

These observations confirm the conclusions drawn from morphological studies described above and the statements by Brown (1930) and Pitelka (1945). They are also in agreement with what is known in other flagellates. In no case have particles been seen to enter the reservoir of any of the Eugleninea, nor indeed of any of the other flagellates. Kent's (1880–2) and Tannreuther's (1923) claims that *Euglena* ingests particles through its reservoir have been proved to be wrong (Mainx, 1928; Hall, 1933*b*). Apparently the reservoir system has some other function than feeding (e.g. respiration or drainage) and when, in the evolution of the Peranemaceae, the mode of nutrition changed to zootrophy, a new opening was developed for the purpose of ingestion. If that be the case, the terms 'cytostome', 'gullet', and 'pharynx' should not be used for the parts of the reservoir system.

DIGESTION

Almost nothing is known of the digestive powers of non-parasitic flagellates, although proteolytic enzymes have been found in cultures of certain species of *Euglena* (Mainx, 1928; Jahn, 1931; Hall, 1937).

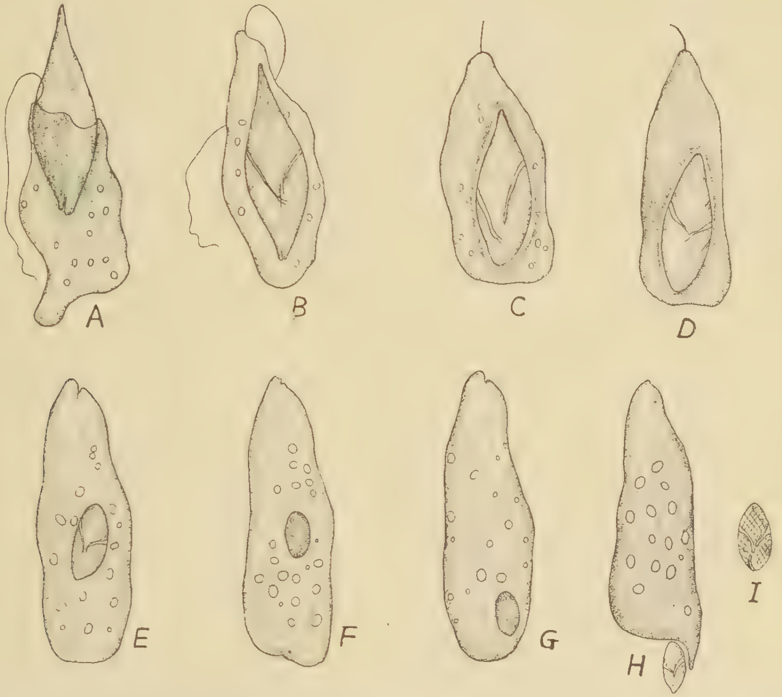
Which of the substances present in the food eaten by *Peranema* are indeed digested can only be ascertained by feeding experiments with different kinds of food. For this purpose *Peranema* from cultures with yeast as food has been used. Since such individuals contain only a moderate amount of reserve substances, observation is relatively easy. The food substances used were: lipoid-free casein, egg-albumin, almond-oil, and rice-starch. To one of two watch-glasses with 2 c.c. of the culture-fluid food was added, while the other one was used as control. At intervals samples were taken for microscopic examination. Hanging drops containing single individuals, which were isolated after having ingested a number of food-particles, were also used. These gave a clearer picture of the changes in the ingested food, because the uptake of new food-particles was prevented. Simple histochemical tests (such as iodine for starch; sudan III, sudan black, and chlorophyll-extract for fats; potassium hydroxide for paramylon) were made.

The food-vacuoles of *Peranema* assume the shape of the ingested food and do not undergo translocation along a constant path within the cytoplasm. They either remain at the same place or are moved at random by body-movements. They are largest when newly formed and decrease in size gradually until they are entirely absorbed; or, more usually, the remains, much reduced in bulk, are discharged through the area of defaecation. Although the digestible parts of the food must have been liquefied in the course of digestion, an enlargement of the food-vacuoles, such as commonly occurs in ciliates and rhizopods, has never been seen.

The digestion of *Euglena viridis* by *Peranema* may be taken as an example (Text-fig. 7). Newly ingested cells preserve their colour for about half an hour and then fade to yellow; no change of size is as yet observable. At the same time a very thin layer of the cytoplasm of the *Peranema* around the *Euglena* becomes transparent. After 1 hour the size of the *Euglena* has decreased to about two-thirds. The transparent layer is still visible. By the next day the *Euglena* has been reduced to about one-fifth its original size. Its shape has become ovoid, its colour brown, and it is no longer recognizable as a *Euglena*. The transparent layer has disappeared, and many paramylon grains are seen in the cytoplasm of the *Peranema*. The remains of the *Euglena* are usually discharged, but sometimes they may remain in the body for another day or two, although no further decrease in size can be detected during this time. The discharged remains seem to consist mainly of the periplast. Striations are still visible, as well as splits, apparently those made by the rod-organ.

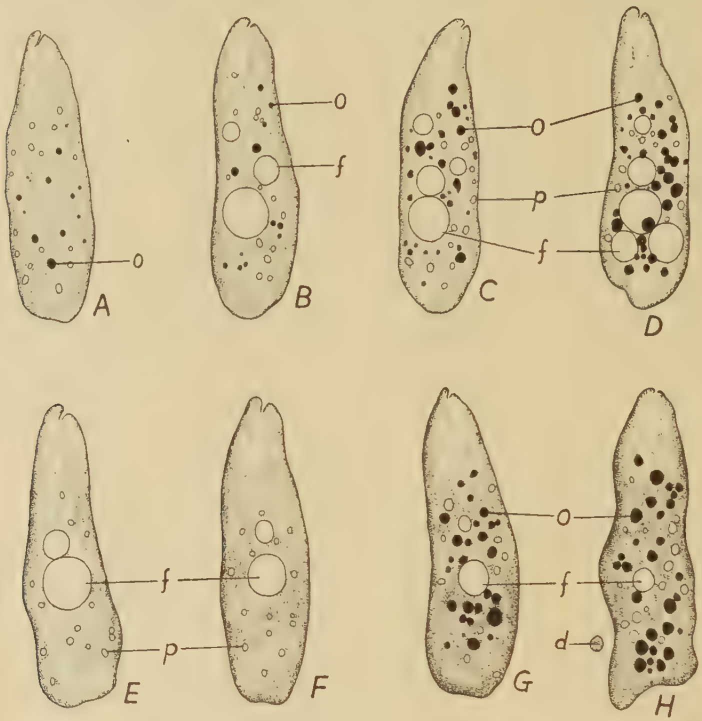
By feeding *Peranema* with yeast cells stained with indicators (Shapiro, 1927, &c.; Pringsheim 1928), the pH of the food-vacuoles was found to lie between 5·8 and 6·8. This is very different from those in ciliates and rhizopods, where the pH can be as low as 3 (Shipley and de Garis, 1925; Shapiro, 1927; Pringsheim, 1928; Mast, 1942; Mast and Bowen, 1944).

While rhizopods and ciliates are known to digest fat (Lund, 1914; Dawson and Belkin, 1928, 1929; Mast, 1938; Wilber, 1942) nothing so far is known in this respect about flagellates. For the relevant experiments almond-oil, stained dark blue with sudan black, was used as food, in order to distinguish the ingested fat from that formed by resynthesis. *Peranema* ingested these stained oil-droplets without visible ill effects (Text-fig. 8). After 2 hours of feeding almost every individual contained a number of blue food-vacuoles. The sudan III test did not reveal any increase in the number of oil-droplets in the cytoplasm. After 5 hours, however, in addition to the large, spherical, blue oil-drops, many droplets, staining red with sudan III, were present in the cytoplasm of most individuals. Five hours later the size and number of colourless droplets stainable with sudan III had markedly increased. In individuals isolated in hanging drops it was found that, while the secondary



TEXT-FIG. 7. Digestion of *Euglena viridis* by *Peranema*. A, *Peranema* engulfing a *Euglena*. B, The *Euglena* is ingested; note the two splits on the surface of the *Euglena* made by the rod-organ. C, After an hour, the size of the *Euglena* has diminished and a thin clear layer appears around it. D, After 2 hours of ingestion. E, The next day, the size of the prey is much reduced, and the clear layer has disappeared; paramylon-granules appear in the cytoplasm of the *Peranema*. F–H, Successive stages on the third day. H, The remains of the *Euglena* are discharged. I, Enlarged view of the discharged *Euglena* with periplast-striation easily visible.

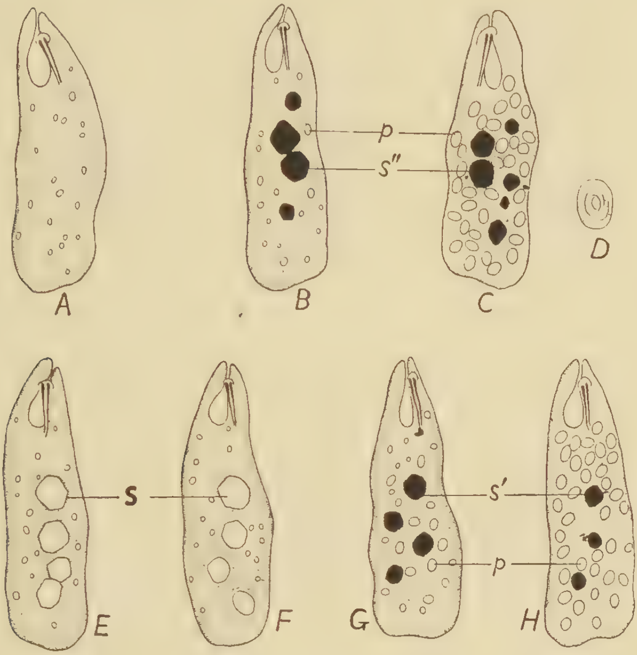
droplets increased gradually, the ingested oil-drops decreased in size. Evidently these colourless droplets are fat-substances resynthesized from products of digestion, rather than formed by splitting up the ingested oil into smaller droplets.



TEXT-FIG. 8. Digestion of almond-oil. A, Control individual not fed with almond-oil. B–D, Individuals which have taken up almond-oil stained blue with sudan black supplied in a watch-glass. B, After 2 hours of feeding. C, After 5 hours. D, After 24 hours of feeding. E–G, Successive stages of individual isolated in hanging drop and digesting ingested oil. E, Two drops of almond-oil have just been ingested. F, After 3 hours; the drops have already decreased in size. G, After 5 hours of ingestion; many oil-droplets have appeared in the cytoplasm. H. Individual after 24 hours of digestion, with a further increase in the number of oil-droplets in the cytoplasm; the remains of one of the ingested oil-drops (*d*) have been discharged into the hanging drop. *f* = food-vacuoles, containing dark-blue oil-drops; *o* = colourless oil-droplets stainable with sudan III; *p* = paramylon granules.

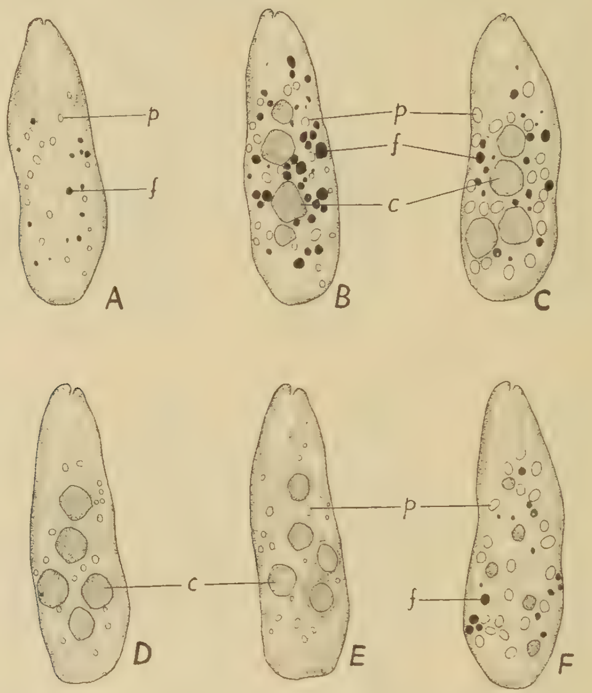
Starch is also digested by *Peranema*, though but slowly (Text-fig. 9). After 1 day's feeding with rice-starch many ovoid paramylon granules appear in individuals which before contained only a few small granules. The newly formed granules are of the same size and shape as those in individuals from milk cultures. After some time, only the centre of the ingested starch-grains stains blue with iodine, while the outer layers are faintly brown. This indicates that the starch is chemically attacked. The decrease in size of the starch grains is clearly observed in hanging-drop preparations. The correlation between increase in number and size of paramylon granules and the decrease in size of the ingested starch shows that the former result from digestion of the latter.

For observations on the digestion of protein, casein was used, mainly because it is easy to handle and has proved a suitable food (Text-fig. 10). It was found that after 24 hours, when the ingested casein particles have decreased to about one-half or one-third of their original size, many oil-droplets of various size appeared; the quantity of paramylon only increased subsequently. In order to decide whether the oil is derived from fatty impurities in the casein, specially re-purified casein was used in repeating the experiment. The result was almost the same. It is concluded, therefore, that *Peranema* is capable of digesting protein and of converting it to oil and paramylon.



TEXT-FIG. 9. Digestion of rice-starch. A, Control individual without starch. B and C, Individuals which have taken up rice-starch in a watch-glass. B, After 1 hour of feeding; starch-grains stain dark blue with iodine. C, After 24 hours of feeding, showing the appearance of numerous paramylon granules; though the starch-grains are represented black in the figure, the peripheral layer in fact only stains faintly, indicating that it is being digested. D, Enlarged view of a starch-grain. E–G, Successive stages of an individual isolated in a hanging drop after ingesting four starch-grains. E, Immediately after feeding. F, After 2 hours of digestion, showing clear layers around two food-vacuoles. G, After 8 hours: paramylon-grains have augmented; one starch-grain stained only faintly in its peripheral layer while the centre was dark blue. H, After 24 hours: many paramylon-grains, starch gradually disappearing. p = paramylon-grains; s = ingested starch; s' = starch blue by iodine; s'' = faintly stained outer layer.

Both oil and paramylon are to be considered as reserve food, because in flourishing cultures, individuals contain more of them, and when the cultures are declining, both decrease in quantity until very little is left, while the size of the cells decreases also. The same changes were observed in individuals specially starved. During one day of starvation the number of oil-droplets increases considerably, but after that both oil-droplets and paramylon-granules decrease steadily in number and size until the individuals die. The



TEXT-FIG. 10. Digestion of casein. A, Control individual not provided with casein. B, Individual fed with heat-denatured but otherwise untreated casein for 24 hours in a watch-glass, showing the appearance of paramylon-grains and many oil-droplets. C, Individual supplied with alcohol-ether extracted casein, after 24 hours, showing a lesser increase in oil-droplets. D–F, Successive stages of an individual digesting extracted casein after isolation in a hanging drop. D, Five food-vacuoles with casein. E, After 2 hours of digestion, showing a thin, clear layer around some of the food-vacuoles. F, After 24 hours, showing decrease in size of casein-particles and increase of paramylon-granules; scattered between them are oil-droplets staining with sudan III. *c* = ingested casein; *f* = oil-droplets; *p* = paramylon-granules.

diminution of the *Peranema* cells is considerable, from the average size of individuals in yeast cultures, 50–55 × 16–18μ, at the beginning, to 42–46 × 9–10μ on the seventh day of starvation. Shortly after that they all perish.

MOVEMENT AND CHEMOTAXIS

Movement

It has long been known that *Peranema* is capable of performing two kinds of movement, gliding along the substratum or the water surface, and swimming freely in the water (Kent, 1880–2; Mast, 1912; Massart, 1920). Although Mast has observed both gliding ('crawling') and swimming movement, he believes that the latter occurs only exceptionally. I have regularly observed free-swimming individuals in undisturbed cultures, however, and can testify that swimming occurs under natural conditions although not as frequently as gliding.

The mechanism of the movement of the *Peranema* flagellum has often been studied (Bütschli, 1883–7; Gray, 1922, 1931; Krijgsman, 1925; Lowndes, 1936, 1941, 1944*a*, *b*; Brown, 1945). But the nature of the gliding movement has not attracted much attention. In the following the term 'flagellum' is used for the long, free flagellum. The function, if any, of the posterior flagellum, attached to the ventral body surface, is unknown. As stated by Massart (1920), *Peranema* always glides on the same (ventral) surface, whether over solid bodies or the water-air interface. During gliding the flagellum stretches out in a straight line and appears rigid and motionless save at the distal end, which beats incessantly to the right as seen under the microscope, its tip describing roughly an ellipse. The change of direction of movement seems not to be determined by the action of the flagellum. In most of my observations the body turned first to the new direction while the flagellum remained behind; only in a few cases did the flagellum bend first. The same conclusion has been reached by Mast (1912).

Mast holds that *Peranema* glides more quickly when the undulation of the flagellum extends over a greater length as a result of irritation. This was not confirmed. On the contrary, when the individuals are stimulated, as Verworn and Lowndes (1944*b*) have already pointed out, though the flagellum beats then throughout its entire length, the body contracts and no longer moves forward. Maximum speed of gliding can only be observed when both the flagellum and the body are in a state of full extension. Measurements at room temperature have given an average speed of 15·6μ/sec. when individuals are moving along the bottom of the container, and 22·9μ/sec. when they are gliding over the water-air interface. Mast (1912) recorded a speed of 21·7–43·4μ/sec., Shortess (1942) 26·17μ/sec., and Lowndes (1944*b*) 20μ/sec., all without mentioning the substratum over which the individuals moved. No correlation has been found between the size and speed of the individuals.

Gray (1931) and Lowndes (1936, 1941, 1944*a*, *b*) believe that the flagellum is an active unit capable of generating its mechanical energy. The latter author stated that the undulation wave starts from the base and is transmitted to the tip. This was confirmed by studying the movement in a soft 0·25 per cent. agar jelly. The fact that only the tip of the flagellum beats strongly suggests also that the flagellum generates at least part of its energy. However that may be, the role of the flagellum in gliding is by no means established. During gliding *Peranema* is attached to the substratum so firmly that it is not readily detached by the sucking force of a capillary tube. Furthermore, when *Peranema* is transferred to a new medium, it is usual for a number of individuals to lose part of their flagellum. Normal gliding was observed in individuals with only two-thirds of the flagellum left, so that the active tip was no longer present. In individuals with still shorter flagella, about one-third of the body-length, normal gliding movement no longer occurs, although they can proceed slowly by wave-like contractions passing from the anterior to the posterior end of the body. Such wave-like contractions can occasionally be observed in normal individuals also. Evidently the cell-body is capable of moving by itself.

The results were confirmed by observations on individuals with the flagellum removed with the aid of a pair of glass needles under a dissecting microscope. When the whole flagellum was removed the individual immediately rounded up and remained motionless, as if dead. After not more than 2 minutes it recovered and, while the body remained in the same place, the anterior end was extended from the mass and then withdrawn by contraction. This was repeated again and again. After 17 minutes a very short flagellum, not longer than one-fifteenth the body-length, had appeared; it could be only seen under high magnification. This short flagellum was for the most part inactive, but occasionally gave a few feeble beats. The individual was still unable to glide. After about half an hour the flagellum had grown longer, to about one-fifth of the body-length. By this time the body was capable of being extended to its full length but never kept this shape for longer than a minute. After 50 minutes the flagellum had grown to one-third of the body-length and was beating forcibly, but stiffly, like a brandished stick rather than like a whip; the body now progressed apparently by peristalsis, waves of contraction being seen to pass from the anterior to the posterior end of the body.

Only after 2 hours, when the flagellum had attained a length equal to that of the body, its end began to undulate in the normal way, and gliding movement was resumed. The results obtained from individuals with only part of the flagellum removed were practically the same; i.e., the body is capable of advancing by peristaltic contractions while the flagellum is still short and seemingly functionless. Gliding, however, only begins when the flagellum has reached almost its normal length, earlier after shedding the flagellum than after its removal by an operation.

All these observations, especially that with half the flagellum shed, support the suggestion that the body is moving independently of the flagellum, which is not essential for gliding. This is comparable to the behaviour of other Euglenineae, e.g. *Euglena mutabilis* Schmitz, which are capable of gliding although devoid of actively functional flagella, only short stumps, not sticking out of the reservoir opening, being present. It is possible that there is in *Peranema* a correlation between the activity of the flagellum and the maintenance of the normal shape of the body. Like other gliding organisms *Peranema* discharges mucilaginous matter which tends to cover the surface of the container of old cultures, even if free from other organisms. What connexion this mucilage may have with the gliding movement is not known in *Peranema* any more than in other instances.

Lowndes's assertion (1944*b*) that the flagellum may have a sensory function is in agreement with my observations. When a gliding *Peranema* hits an obstacle with the tip of its flagellum the latter undulates strongly, and the body contracts spasmodically before it has itself touched the obstacle. Whether this is a grain of sand or suitable prey does not at first make any difference. We conclude that the tip of the flagellum is mechanically irritable, and that the difference between inert particles and food only affects behaviour if the latter produce chemotactically active substances which prevent the gliding individual from turning away. When beginning to glide again, an individual that has hit an indifferent object moves in any direction, without preference; one that has come near to a nutritive object, however, glides nearer and nearer to it. The flagellum is therefore sensitive to touch, while chemical sense may be located in the cell-body.

Whereas the role of the flagellum in the gliding movement is doubtful, swimming is definitely performed by its action. By using a compound microscope with the tube horizontal and fastening a test-tube culture to the vertical stage, the swimming movement can be watched without agitating the culture. As the individuals proceed in all directions at random, the translocation is certainly not due to streaming of the medium. During swimming the body rotates continually, usually counter-clockwise but sometimes also clockwise. Generally the posterior end moves almost steadily in a straight line while the anterior end describes a spiral, the body pivoting about the posterior end, but sometimes the body describes a double cone. The greater, basal part of the flagellum rotates, describing a funnel, also counter-clockwise or clockwise, while its tip is bent sideways and undulates as in gliding. *Peranema* does not swim steadily over a long distance. At short intervals of time the body contracts without apparent cause and, after re-extending, the individual starts swimming again, either in the previous or more frequently in another direction. The speed of swimming of different individuals varies between 20·3 and 42 μ/sec. with an average of 31 μ/sec. (faster than when gliding).

Chemotaxis

The existence of chemotactic irritability in *Peranema* is revealed by the tendency to assemble around sources of food. When for instance a dead *Euglena spirogyra* is introduced into a drop of a *Peranema* culture, many individuals soon assemble around it, and when a piece of casein is hung in a test-tube culture of *Peranema*, it is densely covered with individuals after a few hours. Chemotaxis is, however, not restricted to substances suitable as food. If coarse carmine lumps of the size of about half a wheat grain are added to some drops of *Peranema* culture in a watch-glass, individuals soon assemble around them and proceed to ingest them, piece by piece. It is evident that carmine is not ingested by chance.

For testing chemotaxis the capillary method of Pfeffer (1888) was adapted to the special needs of this organism. Pringsheim and Mainx (1926) in their work on *Polytoma* filled capillaries, left open at both ends, by their own suction force, instead of under the air-pump; but for the present experiments it was necessary to seal one end after filling, by quickly dipping into melted paraffin, because during the relatively slow movement of the organisms the fluid column in the capillary began to move. Another innovation connected with the larger size of the organisms was the use of watch-glasses instead of drops between slide and slip. About 0·2 c.c. of the stock-culture was poured into the watch-glass and left on the stage of the microscope for at least an hour before the open end of the capillary was dipped into its centre.

Very distinct reactions are exhibited towards acetic, nitric, hydrochloric, and sulphuric acids. At certain concentrations, for instance 1/100 n HCl, individuals assemble around the opening of the capillary, forming a ring with a clear space within, owing to equilibrium in this zone between attractive and repulsive action. The higher the concentration, the larger the diameter of the empty space. When the concentration of the acid is decreased, the empty space becomes smaller and eventually, at proper concentrations, the individuals react by directly coming to the opening of the capillary, forming a cluster instead of a ring. Such concentrations are considered to be those to which *Peranema* reacts positively. They have been found to be:

Acetic acid	1/200–1/500 N
Nitric acid	1/750–1/1000 N
Hydrochloric acid . . .	1/650–1/800 N
Sulphuric acid . . .	1/550–1/800 N

The fact that the thresholds of the three strong acids are of the same order and are more dilute than that of the weak acid, and that corresponding salts of these acids do not provoke any reaction of *Peranema*, indicates that the chemotaxis is caused by H^+ ions rather than by the anions. A similar phenomenon is known for *Paramecium caudatum* (Jennings, 1906), *Polytoma uvella* (Pringsheim and Mainx, 1926), *Euglena gracilis* (Mainx, 1928), and *Astasia ocellata* (ibid.).

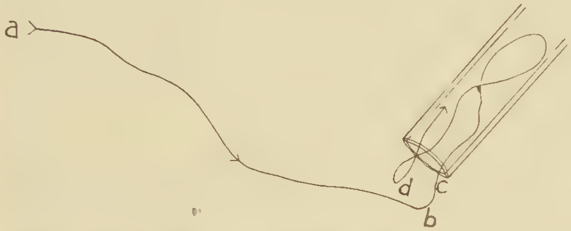
To alkalis no clear reaction was observed. Protein-decomposition products, such as yeast-extract, beef-extract, bacto-tryptone, and bacto-peptone, have a strong attractive effect. The threshold for yeast-extract (dry, Difco) is at 0·1 per cent.; for bacto-peptone at 0·05 per cent.; for the other two substances at 0·001 per cent.; while 0·1–0·2 per cent. of these causes very strong positive chemotaxis.

Starch grains do not exhibit an attractive action when presented in a capillary tube, while carmine, which is engulfed so plentifully, causes strong attraction in these conditions. Even very dilute suspensions of carmine in capillaries cause positive chemotaxis.

The impression that dead individuals of *Euglena* are sought out by *Peranema* with the help of a chemical sense was also tested by the capillary method. *Euglena spirogyra* or *E. viridis*, killed by moderate heating in $CaSO_4$ solution, were sucked into capillaries so that they lay at a short distance from the open end, and the *Peranema* individuals in the watch-glass could not touch them. Many *Peranemae* approached the opening of the capillary, some of them gliding forward until they came near to the dead *Euglena*. Even old culture fluid, freed from *Euglena* by centrifuging, caused positive reaction. As this medium is slightly alkaline, the positive reaction cannot be caused by carbonic acid discharged by *Euglena.* The fresh, unused culture medium is not attractive.

In a similar way it was shown that living or heat-killed *Saccharomyces exiguus* exude chemotactically attractive substances which can be washed out and tested with a positive result.

The mode of reaction can easily be watched, owing to the slowness of movement (Text-fig. 11). It is clear that *Peranema* turns back when about to leave a diffusion zone into which it has penetrated by chance, exhibiting phobo-chemotaxis. Whether the chemotaxis of *Peranema* could be described as klino-kinesis can only be decided by special investigations.



TEXT-FIG. 11. Diagram of the path of a *Peranema* gliding into a capillary tube containing water-extract of carmine. (*a*) starting point from which the individual moved steadily to (*b*), then it turned to the left and approached the opening of the capillary; (*c*) after a few minutes it entered the capillary; when it came out to point (*d*) it turned sharply and went back into the capillary.

I wish to thank Dr. E. G. Pringsheim for his suggestion to use his culture of *Peranema* for elucidating the biology of this organism, and for his advice and encouragement during the two years and a half I worked at Cambridge. Thanks are also due to Dr. V. E. Coslett, Cavendish Laboratory, for his help in electron microscopy, and to Dr. L. E. R. Picken, Department of Zoology, for his introduction into the use of protein dispersing agents for studying the flagellar structure.

REFERENCES

Brown, H. P., 1945. 'On the structure and mechanics of the protozoan flagellum.' Ohio J. Sci., **45**, 247.

—— 1946. 'On the structure and mechanics of the protozoan flagellum.' Abst. Dissert. The Ohio State Univers., **49**, 25.

Brown, V. E., 1930. 'The cytology and binary fission of *Peranema*.' Quart. J. micr. Sci., **73**, 403.

Bütschli, O., 1876. 'Beiträge zur Kenntnis der Flagellaten, &c.' Zeit. wiss. Zool., **30**, 205.

—— 1883–7. 'Protozoa' in Bronn's *Klassen und Ordnungen des Thierreichs*. Bd. 1. Leipzig and Heidelberg (Winter).

Butterfield, C. T., 1929. 'Experimental studies of natural purification in polluted waters. III. A note on the relation between food concentration in liquid media and bacterial growth.' U.S. Publ. Health Repts., **44**, 2872.

Chadefaud, M., 1938. 'Nouvelles recherches sur l'anatomie comparée des Eugléniens: les Péranémines.' Rev. Algol., **11**, 189.

Dawson, J. A., and Belkin, M., 1928. 'The digestion of oils by *Amoeba dubia*.' Proc. Soc. exp. Biol. and Med., **26**, 790.

—— —— 1929. 'The digestion of oils by *Amoeba proteus*.' Biol. Bull., **56**, 80.

Deflandre, G., 1934*a*. 'Sur la structure des flagelles.' Ann. Protist., **4**, 31.

—— 1934*b*. 'Existence sur les flagelles, de filaments latéraux ou terminaux (mastigonèmes).' C. R. Acad. Sci., **198**, 497.

Dellinger, O. P., 1909. 'The cilium as a key to the structure of contractile protoplasm.' J. Morph., **20**, 171.

Doflein, F., 1916. *Lehrbuch der Protozoenkunde*. Jena (Fischer).

Finley, H. E., 1930. 'Toleration of freshwater protozoa in increased salinity.' Ecology, **11**, 337.

Gray, J., 1922. 'The mechanism of ciliary movement.' Proc. Roy. Soc. B, **93**, 104.

—— 1931. *A textbook of experimental cytology*. Cambridge (Univ. Press).

Hall, R. P., 1926. 'Reactions of certain cytoplasmic inclusions to vital dyes and their relation to mitochondria and Golgi apparatus in the flagellate *Peranema trichophorum*.' J. Morph., **48**, 105.

—— 1933*a*. 'The method of ingestion in *Peranema trichophorum* and its bearing on the pharyngial rod ('Staborgan') problem in Euglenida.' Arch. Protistenk., **81**, 308.

—— 1933*b*. 'The question of the ingestion of solid particles by *Euglena*.' Trans. Amer. micr. Soc., **52**, 220.

—— 1934. 'A note on the flagellar apparatus of *Peranema trichophorum* and the status of the family Peranemidae Stein.' Ibid., **53**, 237.

—— 1937. 'Certain culture reactions of several species of Euglenidae.' Ibid., **56**, 285.

—— and Powell, M. N., 1927. 'A note on the morphology and systematic position of the flagellate *Peranema trichophorum*.' Ibid., **46**, 155.

—— —— 1928. 'Morphology and binary fission of *Peranema trichophorum*. (Ehrenb.) Stein.' Biol. Bull., **54**, 36.

Hardin, G., 1942. 'An investigation of the physiological requirements of a pure culture of the heterotrophic flagellate, *Oikomonas termo* Kent.' Physiol. Zool., **15**, 466.

—— 1944. 'Physiological observations and their ecological significance. A study of the protozoan, *Oikomonas termo*.' Ecology, **25**, 192.

Hollande, A., 1942. 'Étude cytologique et biologique de quelques Flagellés libres.' Arch. Zool. exp. gén., **83**, 1.

Hyman, L. H., 1936. 'Observations on Protozoa: I. The impermanence of the contractile vacuole in *Amoeba vespertilio*. II. Structure and mode of food ingestion in *Peranema*.' Quart. J. micr. Sci., **79**, 43.

Jahn, T. L., 1931. 'Studies on the physiology of the euglenoid flagellates. III. The effect of hydrogen ion concentration on the growth of *Euglena gracilis* Klebs.' Biol. Bull., **61**, 387.

James-Clark, H., 1868. 'On the spongiae ciliatae as infusoria flagellata.' Ann. Mag. Nat. Hist., **1**, 250.

Jennings, H. S., 1906. *The behavior of the lower organisms*. New York (Columbia University Press).

Jollos, V., 1925. 'Flagellata.' *Handbuch der Zoologie*, pp. 115 sqq. Berlin und Leipzig (Walter de Gruyter).

KENT, W. S., 1880–2. *A manual of the infusoria.* London (Bogue).

KLEBS, G., 1883. 'Über die Organisation einiger Flagellatengruppen, &c.' Unters. Botan. Inst. Tübingen, **1**, 233.

—— 1893. 'Flagellaten-Studien.' Zeit. wiss. Zool., **55**, 265.

KORSHIKOFF, A., 1923. 'Über den Bau und die Aggregation der Geisseln bei den Volvocales und den Flagellaten.' Arch. russ. Protist., **2**, 195. (German summary.)

KRIJGSMAN, B. J., 1925. 'Beiträge zum Problem der Geisselbewegung.' Arch. Protistenk., **52**, 478.

LACKEY, J. B., 1929. 'Studies in the life-histories of Euglenida. II. The life-cycles of *Entosiphon sulcatum* and *Peranema trichophorum.*' Arch. Protistenk., **67**, 128.

—— 1933. 'Studies in the life-histories of Euglenida. III. The morphology of *Peranema trichophorum.*' Biol. Bull., **65**, 238.

LEMMERMANN, E., 1913. 'Euglenineae' in *Die Süsswasserflora Deutschlands, Österreichs, etc.* Jena (G. Fischer).

LILLY, D. M., 1942. 'Nutritional and supplementary factors in the growth of carnivorous ciliates.' Physiol. Zool., **15**, 146.

LOEFER, J. B., 1931. 'Morphology and binary fission of *Heteronema acus* (Ehrbg.) Stein.' Arch. Protistenk, **74**, 449.

LOWNDES, A. G., 1936. 'Flagellar movement.' Nature, **138**, 210.

—— 1941. 'On flagellar movement in unicellular organisms.' Proc. zool. Soc. Lond., **111**, 111.

—— 1944*a*. 'The swimming of unicellular flagellate organisms.' Ibid., **113**, 99.

—— 1944*b*. 'The swimming of *Monas stigmatica* Pringsheim and *Peranema trichophorum* (Ehrbg.) Stein and *Volvox* sp. Additional experiments on the working of a flagellum.' Ibid., **114**, 325.

LUND, E. J., 1914. 'The relations of *Bursaria* to food. II. Digestion and resorption in the food vacuole and further analysis of the process of extrusion.' J. exp. Zool., **17**, 1.

MAINX, F., 1928. 'Beiträge zur Morphologie und Physiologie der Eugleninen, I, II.' Arch. Protistenk, **60**, 305, 355.

MASSART, J., 1920. 'Recherches sur les organismes inférieurs VIII. Sur la motilité des Flagellates.' Bull. Cl. Sci. Acad. Royale Belgique, séance d'avril 1920, 116.

MAST, S. O., 1912. 'The reactions of the flagellate *Peranema.*' J. Anim. Behav., **2**, 91.

—— 1938. 'Digestion of fat in *Amoeba proteus.*' Biol. Bull., **75**, 389.

—— 1942. 'The hydrogen-ion concentration of the content of the food vacuoles and the cytoplasm in *Amoeba* and other phenomena concerning the food vacuoles.' Ibid., **83**, 173.

—— and BOWEN, W. J., 1944. 'The food-vacuole in the Peritricha, with special reference to the hydrogen-ion concentration of its content and of the cytoplasm.' Ibid., **87**, 188.

—— and HAWK, B., 1936. 'Response to light in *Peranema trichophorum.*' Ibid., **70**, 408.

PFEFFER, W., 1884. 'Lokomotorische Richtungsbewegungen durch chemische Reize.' Unters. bot. Inst. Tübingen, **1**, 363.

—— 1888. 'Über chemotactische Bewegungen von Bakterien, Flagellaten und Volvocineen.' Ibid., **2**, 582.

PITELKA, D. R., 1945. 'Morphology and taxonomy of flagellates of the genus *Peranema* Dujardin.' J. Morph., **76**, 179.

PRINGSHEIM, E. G., 1928. 'Physiologische Untersuchungen an *Paramaecium Bursaria.*' Arch. Protistenk., **64**, 289.

—— 1948. 'Taxonomic problems in the Euglenineae.' Biol. Rev., **23**, 46.

—— and HOVASSE, R., 1950. 'Les relations de parenté entre Eugléneacées et Astasiacées.' Arch. Zool. exp. gén., **86**, 499.

—— and MAINX, F., 1926. 'Untersuchungen an *Polytoma uvella* Ehrbg., insbesondere über Beziehungen zwischen chemotaktischer Reizwirkung und chemischer Konstitution.' Planta, **1**, 583.

RHODES, R. C., 1926. 'Mouth and feeding habits of *Heteronema acus* (The mouth of the Peranemidae Klebs, involving the classification of the Euglenoidina relative to the new family Heteronemidae Calkins).' Anat. Rec., **34**, 152.

SCHAEFFER, A. A., 1918. 'A new and remarkable diatom-eating flagellate, *Jenningsia diatomophaga.*' Trans. Amer. micr. Soc., **37**, 177.

SCHMITT, F. O., HALL, C. E., and JAKUS, M. A., 1943. 'The ultrastructure of protoplasmic fibrils.' Biol. Symposia, **10**, 261.

SHAPIRO, N. N., 1927. 'The cycle of hydrogen-ion concentration in the food-vacuoles of *Paramaecium, Vorticella,* and *Stylonychia.*' Trans. Amer. micr. Soc., **46**, 45.

SHETTLES, L. B., 1937. 'Response to light in *Peranema trichophorum* with special reference to dark adaptation and light adaptation.' J. exp. Zool., **77**, 215.

SHIPLEY, P. G., and DE GARIS, C. G., 1925. 'The third stage of digestion in *Paramaecia*.' Science, **62**, 266.

SHORTESS, G. S., 1942. 'The relation between temperature, light and rate of locomotion in *Peranema trichophorum* in response to changes in temperature.' Physiol. Zool., **15**, 184.

SKUJA, H., 1948. 'Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden.' Symb. Bot. Uppsaliens., **9**, 3.

STEIN, F. R. V., 1878. *Der Organismus der Infusionsthiere*. Abt. III. *Der Organismus der Flagellaten*, Hälfte I. Leipzig (Engelmann).

TANNREUTHER, G. W., 1923. 'Nutrition and Reproduction in *Euglena*.' Arch. Entwick. Mech. **52**, 367.

VLK, W., 1938. 'Über den Bau der Geissel.' Arch. Protistenk., **90**, 448

WILBER, C. G., 1942. 'Digestion of fat in the rhizopod, *Pelomyxa carolinensis*.' Biol. Bull., **83**, 320.

Metachromasy of Nucleic Acids

By L. LISON AND W. MUTSAARS

(*From the Laboratories of Histology and Bacteriology, University of Brussels*)

SUMMARY

If nucleic acids and a metachromatic dye are mixed in amounts giving a low nucleic acid : dye ratio a 'positive' metachromatic effect occurs similar to that observed with truly chromotropic substances: for instance, thionine or toluidine blue become red or reddish-violet. This may be observed in test-tubes with pure solutions of the substances. In microscopical preparations this occurs when the staining is strong.

If the amount of N.A. is such that the ratio N.A. : dye is high, a 'negative' metachromasy occurs, that is, shades of colour are produced that are greener than those obtained in pure water solutions. In microscopical preparations this occurs when the staining of nuclei is very light.

Both 'positive' and 'negative' metachromasy have the characteristics of truly metachromatic phenomena—specific absorption curves and suppression by heat.

EVERY histologist who has had some experience in the use of metachromatic dyes is sure to have noticed how differently the nuclei may appear in the preparations. If they are stained, for example, with toluidine blue, they appear in some cases blue-green or blue, while in other cases they are distinctly metachromatic and stain purple or violet. This metachromatic stain, which has been mentioned incidentally by many authors, is not of the same hue as the stain of the 'true' metachromatic substances, such as mucus or cartilage; the shade is more bluish. For this reason von Möllendorff (1924) proposed to designate nuclei and other elements that have a similar behaviour as 'semi-metachromatic'. We think that a distinction between 'metachromatic' and 'semi-metachromatic' elements, based only upon differences of shades in the staining, is unjustified; for subjective impressions play such a prominent role that every conclusion is subject to discussion. In microscopical preparations stained with toluidine blue, all transitions between a pure blue and a purple can easily be found. Now, where can a line be drawn between orthochromatic, semi-metachromatic, and metachromatic colours? However, the distinction between semi-metachromatic and metachromatic elements is justified when another definition is given: metachromatic elements are those that always stain metachromatically; semi-metachromatic elements are those that stain metachromatically in certain circumstances and orthochromatically in others. Mucus, cartilage, and mast-cell granules belong to the first category and nuclei to the second.

The difference between the shades of the staining of the nuclei is obviously dependent upon the intensity of the staining. When preparations are strongly stained, nuclei are violet; when staining is less intense owing to dilution of the dye-solution or lowering of its pH, the nuclei become more and more

[Quarterly Journal of Microscopical Science, Vol. 91, part 3, September 1950.]

blue. This phenomenon is very similar to the well-known changes of colour of the dye-solution when diluted. However, we were puzzled by the fact that the lightest staining of the nuclei resulted in a green hue which has no equivalent in pure water solutions. Extremely dilute toluidine blue solutions (up to 1 : 1,000,000) are blue, and so also are the alcoholic solutions. The nuclei when only slightly stained are green, with a shade not very different from methyl green staining. Up till now, no explanation has been given of this phenomenon.

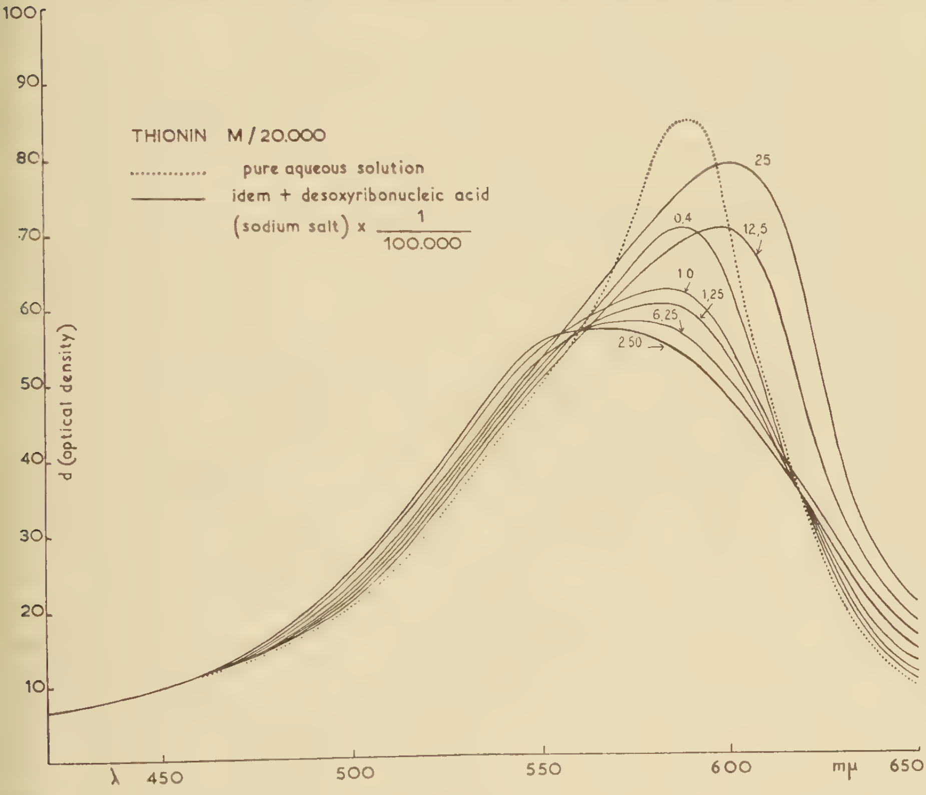
Benazzi-Lentati (1943) showed that cytoplasmic structures containing ribosenucleic acid are metachromatic. According to the author, the metachromasy is due in this case to the presence of metachromatic polysaccharides coexisting with the ribosenucleic acid. It was observed, however, that metachromasy of structures containing ribosenucleic acid disappears with basiphily when they are treated by ribonuclease (Drochmans, private communication), and we may admit that metachromasy can be due to ribosenucleic acid itself. We have observed that structures containing ribosenucleic acid are only metachromatic when strongly stained. Their behaviour is similar to that of nuclei. However, when they are very lightly stained their colour is not so green as the nuclei in the same conditions.

The above-mentioned facts led us to investigate the metachromasy of the nucleic acids. As the factors involved in staining microscopical preparations are numerous and cannot be properly controlled, it seemed preferable to study the action of nucleic acids on pure dye solutions. We owe to the kindness of Prof. Brachet samples of the sodium salts of purified desoxyribose nucleic acid (D.N.A.) and ribosenucleic acid (R.N.A.) from ox pancreas; these salts were soluble without addition of NaOH, giving solutions of pH approximately 5·0.

We have investigated the action of the sodium salts of desoxyribose nucleic acid and ribosenucleic acid, especially upon aqueous solutions of the metachromatic stains, thionine and toluidine blue. When a sodium salt of D.N.A. or R.N.A. is progressively added to dilute (M/20,000) solutions of toluidine blue, the shade of the solution becomes increasingly reddish until the concentration of nucleic acid (N.A.) is about 2·5/100,000. Further addition of N.A. changes the colour to violet-blue and eventually to a blue-green, which is distinctly greener than either alcoholic or aqueous solutions of the dye. Thionine changes from violet to reddish-purple and then to a pure blue. The difference between the violet alcoholic or aqueous solutions of thionine and the blue solution obtained when 20/100,000 N.A. is added is very conspicuous.

The absorption spectra of the solutions have been studied with a Coleman photo-electric spectrophotometer. This has given the following results. When N.A. is added to a 1/20,000 dye solution up to a concentration of 2·5/100,000, the absorption curves obtained are very similar to those that are obtained by adding truly metachromatic substances, such as hyaluronic acid. The characteristic absorption peak of the orthochromatic colour (maximum at 590 mμ for thionin) sinks progressively and a new band arises, with a broad and ill-defined maximum at about 550 mμ. When the concentration of N.A. is further increased this metachromatic band disappears and the maximum absorption-value shifts towards the greater wave-lengths. However, there is no return to the absorption curves of pure aqueous and alcoholic solutions of the dye. The maximum of the new band is located at 605 mμ; furthermore, the extinction-coefficients at any wave-length between 610 and 650 mμ are greater than those of the orthochromatic form of the dye. For instance, at a concentration of 25/100,000 N.A. their values in the region 615–50 mμ are doubled. This increased absorption in the region 615–50 mμ and the displacement of the maximum absorption peak from 590 to 605 mμ explain the greenish shade of the solutions. The behaviour of thionine is shown in the curves of Text-fig. 1. Toluidine blue is very similar, but the absorption peaks are located in longer wave-lengths.

These findings are in accordance with the spectrophotometric curves



Text-fig. 1.

published by Michaelis (1947) in somewhat different experimental conditions. However, some of the conclusions of this author seem to be irrelevant. According to Michaelis, metachromatic dyes are monomeric in alcoholic solutions at any concentration, and also in very dilute aqueous solution, and the absorption curve shows a characteristic α band (maximum absorption for thionine at 590 mμ). In water, at moderate concentrations, the dye is aggregated to dimeres, which show a β band, usually about 30 mμ from the main band. At high concentration, and also in the presence of metachromatic substances, the dye is polymerized, and shows γ bands, closely overlapping, more distant from the main band. Nucleic acid, when in low concentration, would entirely adsorb the dye: this would result in the appearance of the spectrum of a dimeric dye. In our opinion there is only indirect evidence that the dye in the metachromatic condition is dimerized or polymerized; however, this hypothesis is not contradicted by the facts. As we can present no argument for or against the polymerization hypothesis, this point will not be discussed here.

According to Michaelis, N.A. when in high concentration would act in depolymerizing the dyes and in bringing them to the same state as in very dilute water solutions or in alcoholic solutions, i.e. to the monomeric state. Consequently, at high relative concentrations the N.A. should be orthochromatic. This point seems questionable. The absorption spectra of the monomeric orthochromatic form of the dye and those of the dye+N.A. are very different, as shown above. The disappearance of the reddish shade when more N.A. is added to metachromatic dye+N.A. solutions cannot be interpreted as a return to conditions existing in pure water solutions or in nonpolar solvents. A new complex N.A.+dye is formed which exhibits a distinct absorption spectrum.

A further proof of the formation of the new complex is the action of heat. One of us showed long ago (Lison, 1935) that any metachromatic effect is abolished upon heating. This is a striking feature of the metachromatic phenomenon, and can be used for characterizing it. Here is an example. When a trace of a chromotropic substance, such as chondroitin sulphuric acid or heparin, is added to a 1/100,000 solution of toluidine blue the colour changes instantaneously from blue to red. When this red solution is heated it turns progressively purple, violet-blue, and finally, before the boiling-point is attained, it becomes blue, with the same colour as the original pure toluidine blue solution at the same temperature. Upon cooling, the metachromatic hue reappears progressively. All the metachromatic dyes have the same behaviour. The nature of this phenomenon is not yet entirely elucidated, but it is probable that heating loosens the binding of the dye with the chromotropic substance, and so suppresses any metachromatic action.

As expected, a red solution of thionine+*dilute* N.A. turns violet-blue upon heating, and the spectrum is then identical with that of a pure water solution at the same temperature. Now a blue-green solution of thionine+*concentrated* N.A. also turns violet-blue upon heating, and the spectrum is then identical with that of pure aqueous solution at the same temperature. Upon cooling, the first solution becomes red again, the second becomes blue-green again. We may conclude that the blue-green colour imparted to the thionine and toluidine blue solutions when a sufficient amount of D.N.A. is added has the characteristics of a metachromatic phenomenon—specific absorption curve, and suppression upon heating.

We propose for descriptive purposes to term 'positive metachromasy' the shifting of the maximum absorption towards the short wave-lengths (in comparison with the orthochromatic colour), and 'negative metachromasy' the shift towards the long wave-lengths. The common metachromasy exhibited by acid polysaccharides is positive metachromasy. N.A. is positively metachromatic when its concentration is low in relation to that of the dye and negatively metachromatic when it is high.

For the present we cannot define the factors involved in the determination of negative metachromasy. It could be imagined that the phosphoric acid radicle of the N.A. plays some role in this phenomenon. However, the quantitative relationship between the amount of the dye and the amount of N.A. that gives either positive or negative metachromasy is such that no satisfactory explanation can be given. The molecular weight of R.N.A., on the supposition that it is composed of four mononucleotides, is 1,286, or 322 for each nucleotide. Maximum positive metachromasy in M/20,000 thionine solutions is obtained with approximately 3 100,000 N.A. or *circa* M/10,000,000 N.A.; maximum negative metachromasy is obtained with approximately 30/100,000 N.A. or *circa* M/1,000,000. Thus maximum positive metachromasy is obtained when only one phosphoric acid radicle is present for 500 molecules of thionine, and maximum negative metachromasy when one phosphoric acid radicle is present for 50 molecules of thionine. This extreme disproportion between the number of dye molecules and the number of active substance molecules which is necessary to obtain a metachromatic effect is very puzzling. We have observed the same phenomenon with other metachromatic substances. For instance, if hyaluronic acid is progressively added to a M/20,000 toluidine blue solution, metachromasy increases very rapidly when amounts of hyaluronic acid up to 6/100,000 are added; further additions up to 100/100,000, which is the maximum concentration we have used, produce no more changes. If one admits for the minimum molecular weight of the monomeric hyaluronic acid a value of 395 (corresponding to one unit of acetylglucosamine+glucuronic acid), maximum metachromatic effect is obtained when only one molecule hyaluronic acid is present for approximately 300 molecules of toluidine blue.

However difficult the complete theory of metachromasy may appear, the above-mentioned facts explain some appearances observed in stained microscopical preparations. When the ratio dye : nucleic acid is low, the nucleic acids will assume a blue-green colour; for instance, in nuclei if the staining is light, or in spermatozoa heads where the N.A. concentration is particularly high. If, however, the staining is intense, the nuclei and the ribonucleins show positive metachromasy.

Truly metachromatic substances, on the contrary, always show positive metachromasy, even if the ratio dye : substance is low.

REFERENCES

Benazzi-Lentati, G., 1943. Monit. Zool. ital., **54**, 9–10.

Lison, L., 1935. Arch. de Biol., **46**, fasc. 4.

Michaelis, L., 1947. Cold Spring Harbor Symp. on quant. Biol., **12**, 131.

Möllendorff, W. von, 1924. Ergebn. Anat. EntwGesch., **25**, 1.

An Investigation of the Histochemical Technique for the Localization of Acid Phosphomonoesterase

By FRANCES MACDONALD

(From the Department of Zoology and Comparative Anatomy, Oxford)

With one Plate

SUMMARY

1. A method is described for assessing the depth of 'staining' obtained with the acid phosphatase technique and a detailed scheme is given of the standard technique used.

2. It is concluded that the technique specifically demonstrates the activity of acid phosphatase, since 'staining' is abolished in the absence of substrate, in heated sections, and in the presence of fluoride.

3. An investigation has been carried out to determine the extent to which the reaction is affected by altering various stages in the technique.

4. The effect of formalin fixation on the reaction has been investigated.

5. It has been shown that sites in the rabbit medulla having an affinity for the reagents used in the technique differ from the sites at which a precipitate is deposited as a result of enzyme activity.

6. Evidence is presented suggesting that the technique may not demonstrate the true physiological localization of the enzyme.

7. It is suggested that the technique may be of value as a neurohistological method.

INTRODUCTION

IN 1939 Takamatsu and Gomori simultaneously published techniques for the histological localization of a phosphomonoesterase active at pH 9·0, which have subsequently been widely used. Gomori (1941) described a technique for the histochemical localization of a second phosphomonoesterase with a pH optimum of 4·7. Although both methods are based on similar principles—namely, the trapping and precipitation of phosphate ions liberated by enzyme activity by means of a metallic ion, and the subsequent visualization of the site of precipitation by means of an ion exchange reaction followed by the formation of a coloured precipitate—several authors have found results of the second method to be less reliable than those given by the technique for the 'alkaline' enzyme, sections from the same block mounted on the same slide often showing inexplicable differences in 'staining' (Wolf *et al.*, 1943; Lison, 1948). Moreover, some workers have claimed that the technique does not in fact demonstrate the presence of an enzyme at all (Lassek, 1947; Bartelmez and Bensley, 1947). It was therefore clear that a careful investigation would be necessary before the value of the acid phosphatase technique in histochemistry could be determined.

[Quarterly Journal of Microscopical Science, Vol. 91, part 3, September 1950.]

In the experiments described in the present paper, an attempt was made to answer three questions:

(i) Does the technique demonstrate the activity of an enzyme, and, if so, do its properties correspond to those of any enzyme recognized in tissue extracts?

(ii) Are the results obtained with the technique readily reproducible under standard conditions?

(iii) Does the technique demonstrate the true cytological localization of acid phosphatase?

METHODS

The test tissue used in this investigation was central nervous system, this tissue being selected because preliminary experiments showed that the precipitates produced by applying the acid and alkaline phosphatase techniques to such sections were formed at different sites (see below), and also because a sharply localized reaction is obtained with the acid phosphatase technique. Furthermore, by using central nervous system it is possible to select a standard set of cells and to study the reaction developed in them under different conditions. The standard group of cells used for assessing the acid phosphatase technique was the hypoglossal nucleus of the rabbit; these cells are large and are mainly motoneurons. At its caudal and rostral extremities, the nucleus contains relatively few motoneurons, but for a length of roughly 700μ in the region where the central canal opens into the 4th ventricle, the density of these cells is such that successive 15μ transverse sections contain about forty cells on each side. Sections from this area were used exclusively. Rabbits were perfused under ether anaesthesia with (*a*) about 500 ml. of ice-cold 0·9 per cent. saline in order to wash out the blood, followed by (*b*) an equal volume of the fixative used, also chilled to 0° C. A portion of medulla containing the hypoglossal nucleus was then removed, placed in the fixative and transferred to the refrigerator. After fixation for 24 hours, a standard procedure was adopted for the subsequent handling of tissue so that different steps in the technique could later be altered in different experiments. The standard procedure adopted was based on a combination of the Gomori (1941) and Bodian and Mellors (1945) techniques, and is given in Table I.

The following is the principle of this method: phosphate ions liberated from the substrate at the appropriate temperature and pH are trapped by the lead ions present in the incubating mixture and precipitated as lead phosphate; this precipitate is subsequently brought to view by treatment with dilute ammonium sulphide which converts it to black or brown lead sulphide. Such a procedure is obviously more sensitive to small variations in the composition of the various solutions used and to the presence of contaminating substances than are the ordinary techniques for histological staining, and to eliminate this source of variation as far as possible all steps in the technique were carefully standardized. All aqueous solutions were made up with glass distilled water, and the incubating mixture was always made up 24 hours

TABLE I. *Details of Standard Technique*

Fixation and dehydration	Acetone at 0° C.	24 hours
	Acetone at room temperature	8 hours
Clearing	Acetone-benzene (1 : 1)	16 hours
	Benzene	8 hours
Embedding	Benzene saturated with 57° C. wax in 37° C. oven	1½ hours
	Wax (57° C. m.p.)	2 hours (3 changes)
Sectioning within 24 hours of embedding		15μ
Attachment to slides with glycerine and albumen		
Flattening on hot plate		
Drying on to slide		Less than 24 hours
Dewaxing (2 changes of xylene)		10 min.
Absolute alcohol I		1½ min.
0·1 per cent. celloidin in ether-alcohol		½ min.
Absolute alcohol II		1½ min.
90 per cent. alcohol		1½ min.
Distilled water		2 min.
Incubating mixture (see Bodian and Mellors, 1945)		
	Acetate-veronal buffer at pH 4·7 (Michaelis, 1931)	24 ml.
	3·3 per cent. lead nitrate	0·75 ml.
	Distilled water	67 ml.
	3·2 per cent. sodium glycerophosphate (mixture of α and β forms)	8 ml.

The addition of glycerophosphate to the incubating mixture causes a shift of pH to more alkaline values. Before use, the pH was always brought back to 4·7 by the addition of *N*/10 hydrochloric acid.

Time of incubation	4 hours
Temperature of incubation	37° C.
Washing (3 changes of distilled water)	5 min.
2 per cent. ammonium sulphide	1 min.
Washing (running tap-water)	5 min.
Alcohols to xylenes	4 min.
Mount	Canada balsam

before use and was stored at 0° C. The incubating mixture was always allowed to reach the correct temperature before the slides were put into it. Comparisons between different treatments were always made on sections taken from the same block (see below), and slides treated differently at one stage in the technique were, as far as possible, taken through the rest of the process together.

ASSESSMENT OF RESULTS

There have been many reports of the capricious nature of the acid phosphatase technique (Wolf *et al.*, 1943; Lison, 1948; &c.), but in no case has the extent of variability encountered been estimated. In order to assess this variability, and also to compare the effects of different treatments, it became necessary to devise some measure of the depth of 'staining' seen in the hypoglossal motoneurons in different sections. This was done by assessing the degree of blackening of the cytoplasm of the individual cells according to an arbitrary scale analogous to that used by Hogben and Slome (1931). The scale chosen was as follows:

0 . . .	No reaction.
1 . . .	Yellow colour.
2 . . .	Brown colour.
3 . . .	Black colour with the nucleus readily distinguishable.
4 . . .	Black colour obscuring the nucleus, but the structure of the latter just discernible under the high power.

An estimate of 'staining' for any one section was made only from those motoneurons present in which a nucleolus could be seen; motoneurons in which the section did not include a nucleolus were disregarded.

A grade was assigned to the section as a whole by averaging the individual values given to the cells it contained. Since the majority of the cells present normally fell into a single class or into adjacent classes (e.g. in a typical section 30 out of 43 motoneurons were in grade 4, while in an adjacent section 36 out of 43 motoneurons were in grades 3 and 4), it is permissible to calculate a mean depth of 'staining' per section in this way. When a number of cells in a section showed 'staining' intermediate in depth between two of the above classes, half the cells were included in the higher of the two limiting classes and half in the lower. Grading was always done with the same optical arrangement, namely, 2/3 objective and an eyepiece with a magnification of 10 diameters.

TABLE II. *Variability of 'Staining' between and within Blocks*

	Blocks	*1*	*2*	*3*
Slide 1	Section 1 . .	3·3	2·5	4·0
	Section 2 . .	3·2	2·8	4·0
Slide 2	Section 1 . .	3·6	2·4	4·0
	Section 2 . .	4·0	2·4	4·0

Note. In Tables II, IV, VI, VII, the figures given represent the mean depth of 'staining' of all hypoglossal nucleus motoneurons visible within any one section.

It must be emphasized that these numerical values have no absolute significance, the scale chosen being purely arbitrary. A grade of 3·5 assigned to a section does not mean that all cells in that section are 'stained' to a depth halfway between grades 3 and 4, but merely that the mean depth of 'stain' for that section is darker than grade 3 and lighter than grade 4. However, the grading of sections enabled the data to be treated statistically and comparisons to be made between sections treated experimentally in different ways, and also to estimate the extent of the variability of 'staining' encountered among different sections treated in a similar manner.

This was done by comparing the depth of 'staining' obtained under carefully standardized conditions, in hypoglossal nucleus motoneurons in blocks prepared from three separate animals. In each case the block was prepared in accordance with the standard technique. Two slides were prepared from each block and two 15μ sections were mounted on each slide. Slides were treated throughout by the standard technique. The depth of 'staining' in hypoglossal nucleus motoneurons was estimated in every section. The results are given in Table II.

The results of analysing these figures by the technique of the analysis of variance described by Fisher (1946) are given in Table III.

TABLE III. *Analysis of Variance between and within Blocks*

Item	*Sum of squares*	*Degrees of freedom*	*Mean square*	*Variance ratio*	*P*
Variance between blocks	4·54	2	2·27	39·478	<0·001
Variance within blocks	0·03	1	0·30	..	..
Residual	0·46	8	0·058	..	..
Total	5·03	11	..	..	..

Out of a total sum of squares of 5·03, the within-blocks comparison accounts for a variance of only 0·03. Variations in depth of 'staining' between different sections from a single block are thus slight and probably negligible.

However, out of a total sum of squares of 5·03, the between-blocks comparison accounts for a variance of 4·54. Using the residual variance as an estimate of error, the variance ratio can be calculated. When this is done, it can be seen that variance of this amount will only be found as a result of the operation of chance, less than once per thousand trials. Since all the blocks used in this analysis were treated in a standard manner, these variations may reflect a real difference in enzyme activity. It is therefore clear that in investigating the technique, comparisons between the effects of different treatments should be based on sections prepared from a single block.

The precision with which grading can be estimated when the technique is carefully standardized as described can be calculated from the values obtained by treating a number of sections in the standard manner.

15μ sections were cut serially from a single block and two sections were mounted on each of four slides. All slides were carried together through the various stages in the technique, and a mean depth of 'staining' per section was calculated. The results are given in Table IV.

TABLE IV. *Variability of 'Staining' among Sections from a Single Block*

	Section 1	*Section 2*
Slide 1	3·0	3·6
Slide 2	3·6	4·0
Slide 3	3·3	3·2
Slide 4	3·5	3·1

From these figures the total variance was found to be 0·1070. Assuming that the total variance for all sets of observations carried out under standard conditions is the same, then the variance of the difference of the means of two sets each of n observations is $S^2(1/n+1/n)$, and the standard error of the difference is $S\sqrt{(2/n)}$. The number of degrees of freedom involved in such a comparison is $(n-1)+(n-1)$.

It was therefore possible to calculate from these figures that value of the difference of the means of two sets each of two, four, and eight observations which will lie on the limits of significance at the 0·05 probability level. The results are given in Table V.

TABLE V. *Estimate of Precision of Grading*

For a difference of means of two sets of n observations	*Degrees of freedom*	*Standard error difference*	*2 per cent.*
$n = 2$	2	0·1070	0·460
$= 4$	6	0·0756	0·185
$= 8$	14	0·0535	0·115

Reference to Table V shows that at this level a difference in grading of 0·5 can be established with reasonable certainty on a difference between means of grades of two sets of two sections; and a difference in grading of 0·2 can be established with reasonable certainty on the means of grades of two sets each of two sections. These estimates were used in assessing the results of the experiments reported below.

RESULTS

1. *Does the Technique used demonstrate the Activity of an Enzyme?*

An enzyme exists in certain tissue extracts which at pH 4·7 and 37° C. will liberate phosphate ions from monoesters of orthophosphoric acid (Davies, 1934). This enzyme is sensitive to sodium fluoride (Belfanti *et al.*, 1935; Moog, 1944) but does not require the presence of a divalent cation to activate it. In this respect it differs from the alkaline phosphomonoesterase optimally active at pH 9·0 which is fluoride-insensitive, but is said by Roche (1946) to be activated by divalent cations.

Application of the Gomori acid phosphatase technique to sections of rabbit medulla results in the heavy deposition of lead sulphide in the cytoplasm, axons, and dendrites of hypoglossal nucleus motoneurons together with the deposition of a small amount of this substance in the nucleoli and on granular material within the nuclei of these cells (see Pl. I, fig. 1). It therefore became necessary to decide whether this precipitate was the result of the activity of an enzyme similar to that mentioned above or to the operation of unknown factors. This problem was investigated in the following way.

(i) The depth of 'staining' obtained in sections treated according to the standard technique was compared with that of sections incubated in a mixture made up as described above except that the enzyme substrate (sodium glycerophosphate) was omitted. In no section treated in this way was cytoplasmic 'staining' of motoneurons obtained, although the control sections showed heavy and precise 'staining' of cytoplasm, axons, and dendrites. Nucleolar staining and staining of granular material in the nucleus, however, was obtained in the sections incubated in the absence of substrate.

(ii) Sections were incubated in the normal incubating mixture to which sodium fluoride had been added to a final concentration of M/100 and again compared with sections treated in the normal manner. Here also the fluoride-treated sections showed nuclear staining only, whereas the control sections were heavily blackened. It is interesting to note that nuclear staining in fluoride-inhibited sections of central nervous system is clearly shown in Wolf *et al.* (1943, Fig. 2), but these authors make no comment on this appearance.

(iii) Two sets of sections were carried through the various stages of the technique together. One of these, however, was heated to 90° C. for 2 minutes in distilled water immediately before incubation in the same vessel and for the same time as the other set. The unheated set of sections was heavily 'stained'. Cytoplasmic 'staining' in the second set of sections was completely abolished as a result of the heat treatment, although these sections also showed a nuclear reaction.

As a result of these experiments it was concluded that the substance responsible for the reaction was (*a*) heat labile, (*b*) fluoride sensitive, (*c*) dependent on the presence of a monoester of orthophosphoric acid in the incubating mixture in order to exert its effect. All these are properties which may be attributed to an enzyme and are characteristic of the acid phosphomonoesterase found in tissue extracts.

Moog (1943) has described the existence of an acid phosphomonoesterase in the embryo chick which, under the conditions of the Gomori technique, is activated by divalent manganese. To test whether manganese similarly affected the enzyme of hypoglossal nucleus motoneurons an experiment was performed in which sections of rabbit medulla were incubated in the normal incubating mixture to which manganous sulphate was added in concentrations of M/100, M/1,000, M/10,000. The sections incubated in the mixtures containing the two lowest concentrations of manganese showed no difference in depth of 'staining' compared with control sections incubated without manganese. The sections incubated in M/100 manganese were, however, characterized by the complete absence of cytoplasmic 'staining'. It is evident therefore that the enzyme of rabbit hypoglossal nucleus motoneurons differs from that of the chick embryo in that it is apparently not appreciably activated by low concentrations of manganese, while higher concentrations seem definitely to inhibit it.

The presence of intranuclear 'staining' in sections treated by procedures which would be expected to inactivate an enzyme emphasizes the importance of the routine use of control sections in techniques for the histochemical localization of enzyme activity. If control sections in which the enzyme has been inactivated are always included among the slides processed, then comparison between the active and inactive sections will show how much darkening can be caused by factors other than enzyme activity. Ideally several such controls in which enzyme inactivation has been achieved in several different ways should be run with each new tissue investigated. Comparison between Pl. I, fig. 1, a section treated by the standard procedure, and fig. 2, a section heated to 90° C. in distilled water for 2 minutes before incubation, shows that the reaction in cytoplasm, axons, and dendrites only appears in the unheated section.

The acid phosphatase present in hypoglossal nucleus motoneurons appears to be inhibited by manganese. Since the phosphatase active at alkaline pH has been stated to be activated by such cations (Roche, 1946), this may be taken as evidence that the acid and alkaline phosphatase techniques demonstrate the activity of different enzymes. When the alkaline phosphatase technique is carried out on the hypoglossal nucleus no reaction whatever is developed in cytoplasm, axons, or dendrites of motoneurons although intranuclear staining and staining of blood-vessels and of neuroglial elements occurs. Comparison between Pl. I, fig. 3, a section of hypoglossal nucleus treated by the alkaline phosphatase technique, and fig. 1, a similar section treated by the acid phosphatase technique, illustrates the different distribution of precipitates formed in this tissue by the two techniques.

2. *Reliability of the Technique*

To determine the extent to which the reaction is affected by altering different stages in the technique, experiments were performed in which paired sets of sections from single blocks were put through all the stages of the standard technique except that in the case of one set, one step in the technique was varied. After mounting, the mean depth of 'staining' in the two sets of sections was compared. In this way the effect of altering (*a*) the thickness of the sections, (*b*) time of impregnation in wax, (*c*) the duration of storage of embedded material, (*d*) time of incubation, (*e*) temperature of incubation, (*f*) composition of the incubating mixture, and (*g*) composition of the fixative was investigated.

(i) *Thickness of sections.* A comparison of the depth of 'staining' in 10μ and in 15μ sections cut from a single block and incubated together for the standard time of 4 hours, showed that not only did thicker sections 'stain' more intensely but that the collection of cells present 'stained' much more uniformly than in the 10μ sections. This result is consistent with a larger content of enzyme in the thicker sections.

(ii) *Effect of embedding in wax.* Stafford and Atkinson (1948) state that the acid phosphatase activity remaining in paraffin-embedded material is only 5 per cent. of that found in the fresh tissue. Experiments carried out on formol-acetic fixed rabbit medulla (see below) are in accordance with this finding. Frozen sections of formol-acetic fixed medulla show reaction 4 after only 2 hours incubation, while paraffin-embedded sections of this same formol-acetic fixed material only show reaction 1 after as long as 8 hours incubation. Very considerable enzyme inactivation must therefore occur in formol-acetic fixed material during dehydration and embedding in wax. Since it was not possible to cut frozen sections of acetone-fixed material, no comparable experiment could be carried out with tissue fixed in acetone.

(iii) *Time of embedding in wax.* Sections from acetone-fixed blocks impregnated for 24 hours and for 2½ hours in wax showed comparable depths of cytoplasmic reaction, so that increasing the duration of impregnation up to 24 hours does not necessarily cause further appreciable enzyme destruction. However, during long impregnation times considerable cell shrinkage and distortion occur.

(iv) *Storage of wax-embedded material.* It has been stated that alkaline phosphatase activity may rapidly deteriorate when cut blocks are stored at 0° C. and when wax-embedded sections are stored on slides for more than 7 days at room temperature (Danielli, 1946). In the case of acid phosphatase, comparison between sections stored in wax on slides for 14 days with those kept for only 16 hours shows that no appreciable activity is lost during this period. However, once a block has been cut there is a decay of enzyme activity which is detectable after 3 weeks of storage at 0° C.

(v) *Time of incubation.* Increasing the incubation time results, within limits, in an increase in the depth of 'staining'. When sections prepared from a single block are incubated together for 1 hour, 2 hours, 4 hours, and 24 hours and the mean depth of 'staining' is calculated for each section, the following results are obtained: (*a*) 1 hour incubation: 0·6; (*b*) 2 hours incubation: 2·0; (*c*) 4 hours incubation: 2·7; (*d*) 24 hours incubation: such a dense reaction that all cytological detail is obscured (i.e. reaction 4+).

(vi) *Temperature of incubation.* Sections prepared from a single block were carried together through the steps of the technique except that one set was incubated at 60° C. while the second set was incubated at the standard temperature of 37° C. No difference in depth of 'staining' could be detected between these two sets of sections. It is possible that at the higher temperature enzyme inactivation may have counterbalanced the increase in velocity of the reaction which would be expected to occur with increase in temperature.

(vii) *Composition of the incubating mixture.* Various authors have used incubating mixtures containing different concentrations of lead, e.g. Bodian and Mellors (1945) used lead nitrate at a final concentration of M/1,000 while Hard and Lassek (1946) and Wolf *et al.* (1943) used a final concentration of about M/100. An experiment was carried out to test the effect of varying the concentration of lead. Sections of rabbit medulla were incubated in the standard mixture in the presence of M/100, M/1,000, and M/10,000 lead nitrate. The results are given in Table VI.

It is of interest to note that in the presence of M/100 lead nitrate, those few motoneurons which did 'stain' showed a precipitate in the cytoplasm only but none in axons and dendrites, while motoneurons incubated in the presence of

M/1,000 lead nitrate showed the usual reaction in cytoplasm, dendrites, and axons.

TABLE VI. *Effect of Pb on Acid Phosphatase Reaction*

		M/100 lead nitrate	*M/1000, lead nitrate*	*M/10,000 lead nitrate*
Slide 1	Section 1	0·1	4·0	0·0
	Section 2	0·7	4·0	0·0
Slide 2	Section 1	0·2	4·0	0·0
	Section 2	0·4	4·0	0·0

This shows that in the presence of M/100 lead nitrate considerable enzyme inhibition occurs, a result comparable to that obtained with manganese; a concentration of M/10,000 lead nitrate is presumably too low to allow the precipitation of lead phosphate; M/1,000 lead nitrate is the best concentration of the three tested.

These results accord well with those of Gomori (1941) who made quantitative determinations by biochemical methods of prostatic acid phosphatase activity in the presence of increasing concentrations of lead and found that increasing the concentration of lead nitrate from M/200 to M/50 caused a 35 per cent. inhibition of the enzyme.

(viii) *Composition of the fixative.* Acetone is such a notoriously poor fixative that an effort was made to find some fixative which would be more satisfactory from the cytological point of view but which would cause no more enzyme inactivation than did acetone. Acetone-fixed sections were immersed in solutions of a number of fixatives for 24 hours and were then incubated for 4 and 24 hours.

TABLE VII. *Effect of Fixatives*

Fixative	*4 hours incubation*	*24 hours incubation*
Saturated picric acid	0·0	0·0
Anhydrous pyridine	1·0	1·0
10 per cent. pyridine	1·0	1·0
Potassium dichromate	0·0	Variable 1–4
Absolute alcohol	0·0	4·0
10 per cent. formaldehyde + 1 per cent. sodium chloride	1·5	3·2
4 per cent. formaldehyde + 1 per cent. sodium chloride	3·4	4·0
1 per cent. acetic acid	3·3	4·0
Acetone (control)	3·5	4·0

Four per cent. formaldehyde and 1 per cent. acetic acid are the only fixatives in the range tested which do not inactivate the enzyme in acetone-fixed sections of nervous tissue. Formol-acetic is an excellent fixative for such tissue and was used to fix by perfusion. Frozen sections of spinal cord and of medulla cut from a formol-acetic fixed block showed reaction 4 in the cytoplasm after only

2 hours incubation. However, as has already been stated, formol-acetic fixed material dehydrated through a graded series of acetones and then embedded in paraffin wax shows only reaction 1 after as long as 8 hours incubation; after 24 hours incubation reaction 4 is developed. However, 'staining' is very much more clear cut than in sections from an acetone-fixed block incubated for the same length of time.

Smith (1948) has found that a positive reaction for acid phosphatase can be obtained from nervous tissue fixed in 20 per cent. formalin in Ringer's solution. The experiment reported above suggests that 10 per cent formaldehyde causes considerable inhibition of acid phosphatase, and Smith does state that longer incubation times are necessary after formalin fixation than following acetone fixation but gives no details of the incubation times used. It will be shown later that movement of some substance involved in the reaction demonstrating the activity of the enzyme does occur during lengthy incubation times so that any procedure which involves prolonging the duration of incubation should be avoided.

3. *Cytological Localization*

The precipitate of lead sulphide is produced in the acid phosphatase technique by means of a series of chemical reactions, and before the localization of such a precipitate can be considered to demonstrate the true cytological localization of the enzyme it must be shown that (*a*) the sites at which lead sulphide is deposited are not simply sites having an affinity for the reagents used in the technique, and (*b*) neither the enzyme nor any of the reaction products involved in the technique is capable of moving about within the tissue.

(i) *Selective affinity for lead nitrate.* Sections prepared in the standard manner were dewaxed and immersed in 3·3 per cent. lead nitrate solution for 5 minutes, washed and then treated with 2 per cent. ammonium sulphide, this being the method used to render the white precipitate of lead phosphate visible. No reaction developed in cytoplasm, axons, or dendrites of material treated in this way but the nucleolus and scattered granular material in the nucleus showed a reaction. Clear and precise staining of the capillary bed was also obtained (see Pl. I, fig. 4, and compare with fig. 1).

(ii) *Selective affinity for sodium glycerophosphate.* Sections which have been heated to 90° C. for 2 minutes in distilled water and then placed in the normal incubating mixture show a similar intranuclear reaction but a complete absence of cytoplasmic 'staining', so that neither sodium glycerophosphate nor the constituents of the buffer have any affinity for cytoplasmic sites.

The sites in motoneurons having an affinity for the reagents used in the technique differ from the sites of enzyme activity.

(iii) *Possibility of sites having an affinity for lead phosphate.* Sections were prepared by the standard technique and were treated in the following manner:

(*a*) Firstly, lead phosphate was precipitated over heat-inactivated sections by non-enzymic procedures: (1) rapidly by mixing disodium hydrogen phosphate and lead nitrate on a slide, and (2) slowly by adding hydrogen peroxide to the normal incubating mixture to a final concentration of 10 per cent. All such sections showed, after treatment with ammonium sulphide, yellow background 'staining' and a dense precipitate which covered both the sections and those portions of the slide which had been coated with wax. There was no evidence of any localization in or on cells whatsoever.

(*b*) Secondly, heat-inactivated sections of rabbit medulla were mounted on slides and an active section was superimposed upon each. The slides were then incubated in the standard manner but for 24 hours, and the distribution of the resulting precipitate was carefully investigated. It was found that the upper (active) section showed a dense deposit of reacting material in the cytoplasm, axons, and dendrites, while the lower (inactive) section showed no cytoplasmic precipitate.

Danielli (1946) and Jacoby and Martin (1949) have both carried out experiments of this type with the alkaline phosphatase technique. In Danielli's experiments active sections were superimposed upon heat-inactivated sections of the same tissue, while in Jacoby's experiments sections of a tissue known to be rich in alkaline phosphatase activity were superimposed upon sections of a tissue giving a negative reaction with the technique. In Danielli's experiments no precipitate appeared in the inactive sections, while in Jacoby and Martin's experiments a nuclear reaction appeared in those tissue sections said to contain very little alkaline phosphatase. This discrepancy in results could be explained by assuming that in Jacoby and Martin's experiments a very small amount of enzyme is present in the negatively reacting tissue, and that this is activated by the diffusion of some substance from the positively reacting section.

(iv) *Sites having an affinity for phosphate.* Sections prepared in the standard manner were dewaxed and soaked in sodium dihydrogen phosphate, washed, immersed in 3·3 per cent. lead nitrate solution, washed again, and subsequently treated with 2 per cent. ammonium sulphide solution. Sections treated in this manner showed the intranuclear reaction together with clear and precise staining of the capillary bed but a complete absence of cytoplasmic staining.

(v) *Movement of lead phosphate.* Sections from a single block were prepared and incubated according to the standard technique. After incubation, i.e. after the deposition of lead phosphate, one set of sections was washed in distilled water for the standard time of 5 minutes while the second set was washed for 24 hours. Both sets of sections showed a mean reaction of 3·0 (see Pl. I, figs. 5 and 6). Since no decrease in depth of 'staining' can be detected after sections have been washed for as long as 24 hours, it is improbable that under the conditions of the Gomori technique, lead phosphate can be moved about in sections of rabbit medulla once it has been deposited. Further, if no detectable redistribution occurs during a 24-hour washing period, it is most unlikely that any movement will occur during the standard washing period of 5 minutes.

Bartelmez and Bensley (1947), however, find that washing teased acetone-fixed peripheral nerve in distilled water for 16–24 hours either before or after incubation, leaves the axons 'stained' but removes the stainability from the nuclei. This suggests that in some tissues, both the enzyme and lead phosphate can be washed out of certain sites. However, by the methods described here, it has been impossible to demonstrate either that there are sites in the rabbit medulla having an affinity for phosphate or that lead phosphate can move about within this tissue. There is no reason to suppose that in rabbit medulla the reaction product is attracted away from those sites at which it is formed.

(vi) *Possible movement of enzyme.* (*a*) Sections prepared from a single block were washed in distilled water before incubation for (1) 24 hours, and (2) the standard period of 5 minutes. All sections were then treated by the standard technique. Sections which had been washed for 5 minutes showed reaction 3·0 (see Pl. I, fig. 5) in the cytoplasm, while those washed for 24 hours showed reaction 2·0 (see Pl. I, fig. 7). This suggests that either the enzyme or possibly a co-enzyme or activator may diffuse out of sections during prolonged periods of washing. The possibility that acid phosphatase can be leached out of acetone-fixed sections of peripheral nerve has been mentioned by Bartelmez and Bensley (1947).

(*b*) If movement of some substance involved in the reaction can occur during prolonged periods of washing, it may also take place during lengthy incubation. Since sections incubated for long periods at 37° C. are normally so densely 'stained' that all cytological detail is obscured, sections were incubated at 0° C. in order to slow down enzyme activity and also to allow the maximum time for possible movement of substances. Sections prepared from a single block were incubated at 0° C. for (1) 24 hours, and (2) 48 hours. In the sections incubated for 24 hours, the reacting substance was distributed evenly throughout the cytoplasm of all motoneurons present. In the sections incubated for 48 hours, all motoneurons showed peripheral concentrations of darkly 'staining' material while the central areas of the cytoplasm were paler than in 24-hour incubated sections (see Pl. I, fig. 8).

(*c*) Sections prepared in the normal manner were then incubated (1) for 48 hours at 0° C. followed by (2) 4 hours at 37° C. Sections treated in this way showed a dense reaction throughout the cytoplasm although peripheral concentrations, similar to those seen in sections from the same block incubated for 48 hours at 0° C., were detectable.

On the basis of these experiments, it is not possible to decide whether the substance which moves is an enzyme, an enzyme activator, or a co-enzyme. Furthermore, the movement which occurs cannot be a simple diffusion into or out of the cells. However, the experiments do show that redistribution of some substance involved in the reaction may occur during long incubation periods. Thus great caution should be exercised in accepting the localization of precipitates obtained after long incubation times as indicating sites of enzyme activity, and the claims made by Dempsey and Deane (1946) that the localization of precipitates obtained after as long as 72 hours incubation represents the true physiological position of the enzyme, must be treated with reserve.

Moreover, Smith (1948) publishes figures of formalin-fixed motoneurons showing the precipitate localized in a ring round the nucleus. Since no exact information is given in this paper as to the duration of the 'long' incubation times used, it is doubtful whether this appearance can be regarded as giving an indication of the true physiological localization of acid phosphatase.

DISCUSSION

Some workers have questioned the validity of the acid phosphatase technique on the grounds that a positive reaction can be obtained under conditions in which it is unlikely that an enzyme could remain active. For example, Lassek (1947) claims that he can demonstrate a positive reaction in the peripheral nerves of the cat in sections which have been (*a*) boiled for 1 hour, (*b*) immersed in solutions of widely varying pH, and (*c*) immersed in mercuric chloride. Similarly, Bartelmez and Bensley (1947), also working on peripheral nerve, claim that lead is specifically adsorbed by injured nerve material after it has been boiled. However, neither Lassek nor Bartelmez and Bensley compare the localization of the precipitate in material treated by the standard technique and in similar material subjected to these drastic treatments. It is perfectly possible that the reagents used in the Gomori technique may take part in reactions involving the activity of acid phosphatase and also react with other substances in the tissues or with substances released in the tissues by boiling. Comparisons should always be made between the localization and density of the precipitate in active and in inactive sections before drawing conclusions as to the distribution of acid phosphatase in any given tissue. Precipitates developed in active sections only may be safely considered to be produced as a result of enzyme activity. The results obtained in this investigation support Gomori's claim that the technique demonstrates the activity of a phosphomonoesterase active at acid pH.

However, it seems improbable that the technique demonstrates the true physiological position of the enzyme. Experiments reported above have shown that the localization of the lead sulphide precipitate can be altered by changing the conditions under which the technique is carried out, for example, by altering the time of incubation. Furthermore, since movement of either the enzyme or of some substance associated with it can occur during lengthy incubation and washing, it is possible that it may also take place during fixation and dehydration. However, by cutting frozen formol-acetic fixed sections, it is possible to limit the period intervening between death of the animal and incubation of sections to 3 hours, and when this is done, the localization of the precipitate in motoneurons is similar to that seen in acetone-fixed material treated by the standard method. However, this objection to the method may be more serious when tissues other than medulla are used.

Since a constant positive reaction is obtained in cytoplasm, axons, and dendrites of motoneurons while under the same conditions no acid phosphatase is demonstrable in the nuclei, it seems that acid phosphatase is present at extranuclear sites in motoneurons. Smith (1948) states that in formol-fixed material the nucleus stains more darkly than the cytoplasm. No such phenomenon has been noted during this investigation, but it is possible that the localization of the precipitate differs in material fixed in 20 per cent. formalin and incubated for 'long' periods, from the localization in acetone-fixed or 4 per cent. formol-acetic fixed material incubated for from 2 to 4 hours.

Although the technique cannot be used to decide with any certainty the true physiological localization of acid phosphatase, it has considerable value as a histological method when carefully standardized. Many methods used for staining nervous tissue are capricious, complicated, and difficult to carry out. The carefully standardized acid phosphatase technique, however, as applied to mammalian medulla does show histological detail with remarkable precision. This is well illustrated by Pl. I, fig. 9, a motoneuron of the cat treated by the standard technique. The cytoplasmic reaction in this case is slight, but axons and dendrites have reacted strongly and it is possible to distinguish what are probably *boutons terminaux*. The technique thus deserves further trial purely as a neurohistological method where it may prove to be of considerable value.

REFERENCES

Bartelmez, G. W., and Bensley, S. H., 1947. Science, **106**, 639.
Belfanti, S., Contardi, A., and Ercoli, A., 1935. Biochem. J., **29**, 517.
Bodian, D., and Mellors, R. C., 1945. J. exp. Med., **81**, 469.
Danielli, J. F., 1946. J. exp. Biol., **22**, 110.
Davies, D. R., 1934. Biochem. J., **28**, 529.
Dempsey, E. W., and Deane, H. W., 1946. J. cell. comp. Physiol., **27**, 159.
Fisher, R. A., 1946. *Statistical Methods for Research Workers* (Oliver & Boyd).
Gomori, G., 1939. Proc. Soc. exp. Biol., **42**, 23.
—— 1941. Arch. Path. Lab. Med., **32**, 189.
—— 1943. J. biol. Chem., **148**, 139.
Hard, W. L., and Lassek, A. M., 1946. J. Neurophysiol., **9**, 121.
Heinzen, B., 1947. Anat. Rec., **98**, 193.
Hogben, L., and Slome, D., 1931. Proc. Roy. Soc. B, **108**, 10.
Jacoby, F., and Martin, B. F., 1949. Nature, **163**, 875.
Lassek, A. M., 1947. Stain Tech., **22**, 133.
Lison, L., 1948. Bull. hist. Tech. micr., **25**, 23.
Michaelis, L., 1931. Biochem. Z., **234**, 139.
Moog, F., 1943. J. cell. comp. Physiol., **22**, 95.
—— 1944. Biol. Bull. Wood's Hole, **86**, 51.
Perlmann, G. E., and Ferry, R. M., 1942. J. biol. Chem., **142**, 513.
Roche, J., 1946. Helv. chim. Acta, **29**, 1253.
Smith, W. K., 1948. Anat. Rec., **102**, 523.
Stafford, R. O., and Atkinson, W. B., 1948. Science, **107**, 279.
Takamatsu, H., 1939. Tr. Soc. path. jap., **29**, 492.
Wolf, A., Kabat, E. A., and Newman, W., 1943. Amer J. Path., **19**, 423.

EXPLANATION OF PLATE I

Fig. 1. Rabbit hypoglossal nucleus. Standard acid phosphatase technique. Strong reaction in cytoplasm, axons, and dendrites of motoneurons.

Fig. 2. Rabbit hypoglossal nucleus. Section heated to 90° C. for 2 minutes in distilled water, then treated by the standard acid phosphatase technique. No reaction in cytoplasm, axons, or dendrites of motoneurons. Note slight nuclear reaction.

Fig. 3. Rabbit hypoglossal nucleus. Alkaline phosphatase technique, 24 hours incubation

No reaction in cytoplasm, axons, or dendrites of motoneurons. Reaction in glia and blood-vessels. Note nuclear reaction.

Fig. 4. Rabbit hypoglossal nucleus. 3·3 per cent. lead nitrate followed by 2 per cent. ammonium sulphide. No reaction in motoneurons. Staining of capillary bed.

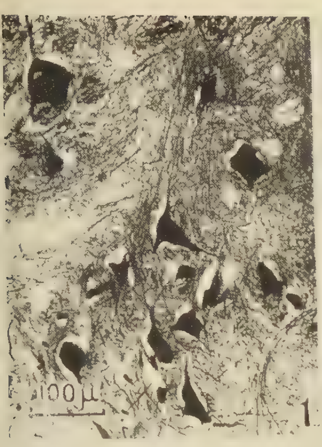
Fig. 5. Rabbit hypoglossal nucleus. Standard acid phosphatase technique. Mean reaction 3 in cytoplasm. (Sections in Figs. 5, 6, and 7 taken from a single block.)

Fig. 6. Rabbit hypoglossal nucleus. Acid phosphatase technique; section washed for 24 hours after incubation. Mean reaction 3 in cytoplasm. (Sections in Figs. 5, 6, and 7 taken from a single block.)

Fig. 7. Rabbit hypoglossal nucleus. Acid phosphatase technique; section washed for 24 hours before incubation. Mean reaction 2 in cytoplasm. (Sections in Figs. 5, 6, and 7 taken from a single block.)

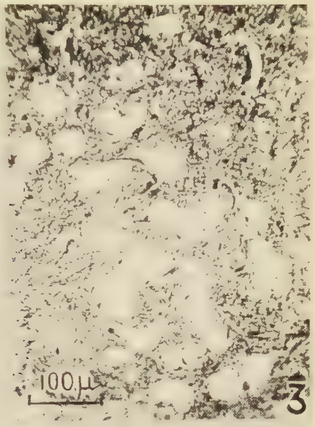
Fig. 8. Rabbit hypoglossal nucleus. Acid phosphatase technique; sections incubated for 48 hours at 0° C. Marked peripheral concentrations of reacting material.

Fig. 9. Mosaic photograph of motoneuron from cat medulla. Standard acid phosphatase technique. Strong reaction in axons. Possible *boutons terminaux* can be distinguished.



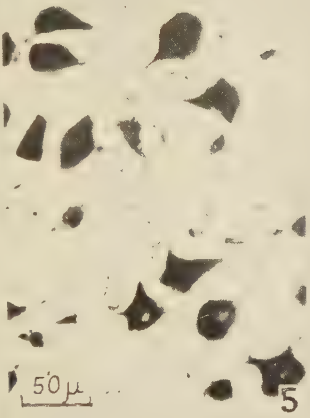
1

2

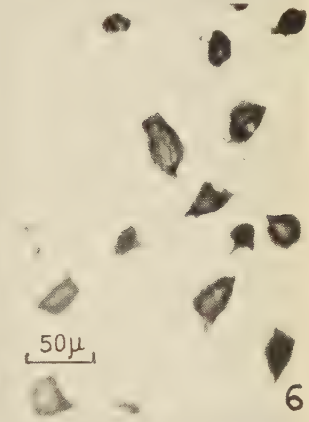


3

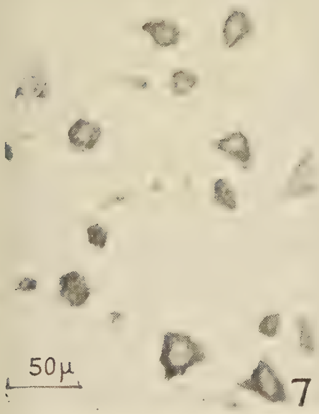
4



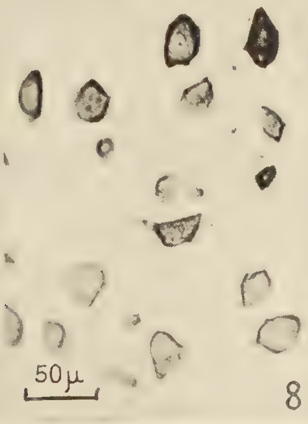
5



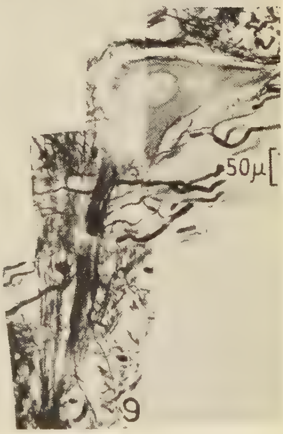
6



7



8



9

F. MACDONALD—PLATE I

A Review of the Methods available for the Determination of the Types of Forces Stabilizing Structural Proteins in Animals

By C. H. BROWN

(*Animal Reproduction Unit, School of Agriculture and Zoological Laboratory, Cambridge*)

SUMMARY

The types of linkages holding protein chains together are discussed, and methods described for determining the types of linkage in animal structural proteins. A short account is given of the results of such tests applied to a variety of invertebrate and vertebrate structures.

INTRODUCTION

IF an animal is small and lives in an aqueous environment of constant composition, the necessary mechanical support and protection for its soft internal structures can be provided by skeletal materials of moderate stiffness and of moderate chemical stability. For larger animals, living in less constant aquatic environments or on dry land, it becomes necessary to provide more rigid support for the soft parts, and external membranes capable not only of preventing mechanical injury to soft tissues beneath, but also giving protection against osmotic changes and against desiccation, without themselves undergoing change as a result of the varying ionic content of the external medium, or as a result of humidity changes. The parasitic mode of life, or periodic exposure to drought in the case of aquatic or subaquatic organisms, or prolonged embryonic development, also necessitate the elaboration of protective and resistant surface membranes, cysts, and egg-shells. In many instances these requirements have been met by the development of membranes composed of proteins. The factors on which the mechanical and chemical properties of these depend can be ascertained by applying to their study certain methods which will be described here. The significance of the results obtained by these methods is only clear against the background of an elementary knowledge of the structure and properties of proteins.

I. *The Structure and Properties of Proteins*

All proteins are polypeptides built up of amino-acid residues united in linear series by peptide links between the amino and carboxyl groups of successive residues, so that a regularly repeating sequence of two carbon atoms and a nitrogen atom forms the backbone of a chain, while the remainder of each amino-acid residue extends laterally, at right angles to the backbone, to form side chains. Such polypeptide chains carry a number of chemically

[Quarterly Journal of Microscopical Science, Vol. 91, part 3, September 1950.]

active groups which determine the solubility properties of the polypeptides. At each peptide link along the molecular backbone, for example, there is a $>C=O$ and a $>N—H$, while the side chains may also carry polar groups, that is, electrically asymmetrical groups with an affinity for each other and for water.

In the so-called globular proteins the polypeptide chains are folded to form approximately spherical molecules. Many of these globular proteins dissolve in water or salt solutions to give solutions of relatively low viscosity even at quite high concentrations. The behaviour of such solutions is controlled by the pH and the salt content of the solvent; they act as bases towards acids and as acids towards bases. At the iso-electric point ionization of the molecule is minimal and the protein is electrically neutral.

Most globular proteins readily undergo denaturation: that is, the spherical molecules become unfolded to form long, fibrillar molecules; if they are denatured at their iso-electric points the proteins become insoluble. In many cases denatured proteins can be redissolved in acids and alkalis; but in contrast to the globular proteins they yield solutions of high viscosity even at very low concentrations of protein.

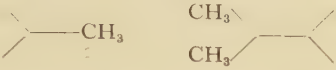
Structural proteins from skeletal tissues, various protective membranes and natural fibres (from hair and feathers) are for the most part in the denatured, fibrillar state. In membranes, the protein molecules are frequently oriented with their backbone in the plane of the membrane while in fibres they are oriented more or less parallel to each other.

The behaviour of fibrillar proteins depends upon the nature of the bonds established between adjacent molecules, which, in turn, depends on the nature of the active groups present in the side chains. If the proteins are built up from amino-acid residues with short side chains, the molecules can approach each other closely and intermolecular attractions may develop between the backbones. Such protein structures are stiff and have a relatively high modulus of elasticity and low reversible extensibility. If, on the other hand, the proteins contain amino-acid residues with long side chains, it is not possible to pack the chains closely together; intermolecular attractions develop only between the polar or other groups at the end of the side chains, and a structure results of lower strength but with greater extensibility.

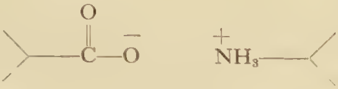
II. *Types of Linkage between Adjacent Protein Molecules*

The linkages holding protein chains together may be roughly divided into three kinds: Van der Waals attractive forces, electro-valent linkages, and covalent linkages (Meyer, 1942; Frey Wyssling, 1948).

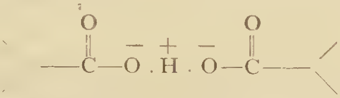
Van der Waals forces are those which hold non-polar molecules (e.g. paraffin molecules) together, and would presumably exist between terminal $—CH_3$ groups in protein side chains. They may be regarded as feeble attractions between groups which, considered over any length of time greater than 10^{-20} seconds, are electrically neutral; but as a result of the development of temporary, local concentrations of charge in atoms of adjacent molecules, as the electrons revolve in their orbits, they feebly attract each other over short periods of time.



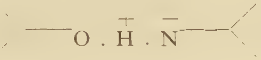
Electrovalent linkages depend on electrostatic attractions between positively and negatively charged polar groups as, for example, between ions in salt crystals. The salt link between adjacent $-NH_3^+$ and $—COO^-$ groups in protein side chains is of this type:



The hydrogen bond may be considered as a special case of the electrovalent bond, resulting from the attraction of two negatively charged atoms to a common hydrogen ion, as, for instance, between two $—COO^-$ groups of adjacent molecules:



or between imino and carbonyl groups of adjacent protein backbones:



The strength of electrovalent links is considerably influenced by the ionic content of the medium surrounding the protein; and fibrous proteins held together solely by these linkages only maintain constant properties in a constant environment.

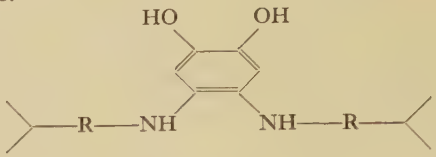
Covalent linkages result from the sharing of electrons by the atoms at the point of linkage. In the —S—S— bond of cystine, two electrons are shared between the two sulphur atoms forming the link:

$$>CH—CH_2—S—S—CH_2—CH<$$

This sort of bonding is much more independent of the ionic content of the medium than is the electrovalent bond, and requires greater expenditure of energy to break it than is required to break an electrovalent bond. (The bond energy of an —S—S— bond is 63·8 k. cal./mol. as opposed to approximately 5 k. cal./mol. for hydrogen bond.) If, therefore, a fibrillar protein is held together for the most part by covalent bonds it is a very stable structure.

It was Speakman (1936) who first offered a satisfactory explanation of the significance of the high cystine content of keratins, suggesting that the cystine formed bridges between adjacent molecules, thus linking them by stable covalent bonds, and so producing the stiffness and high chemical stability characteristic of these proteins.

It is also possible to increase the stability of fibrillar proteins by the action of tanning agents. In general, these are substances whose molecules are able to establish a number of links, either electrovalent or convalent, between themselves and the protein chains. The vegetable tannins, used industrially in the tanning of leather, form electrovalent links for example; while formaldehyde and o- or p-benzoquinone are typical representatives of the group which form covalent bonds.



The tanning agent converts the proteins into a form in which they swell less and exhibit greater mechanical strength and rigidity.

The demonstration of tanning as a biological process was due to Pryor (1940) who showed that the hardening of the protein oothecae of cockroach is brought about by the addition to the protein of an orthodiphenol (protocatechuic acid, Pryor, Russell, and Todd, 1946), which on oxidation by an enzyme to orthobenzoquinone forms covalent links between itself and the protein.

III. *Determination of Types of Linkage present in Structural Proteins*

Theoretical considerations. When some or all of the cross linkages present in a fibrous protein are broken, the molecular chains tend to curl up and shorten, since they are no longer held together by intermolecular forces. Water molecules and ions from the surrounding medium then penetrate between the chains, pushing them apart, causing the structure to swell and, if all the linkages are broken, finally to dissolve. The type of swelling that takes place is dependent on the spatial arrangement of the protein molecules. Tissues in which the molecules are arranged in unoriented networks swell equally in all directions (isotropic swelling), while oriented structures always exhibit anisotropy of swelling. In membranes with molecules oriented parallel to the plane of the membrane, maximum swelling occurs in a direction at right angles to the molecule backbones, the membrane increasing mainly in thickness. In fibres formed from parallel protein molecules, the reagent may only penetrate into the interfibre space, causing the fibres to increase in thickness without change in length. If the reagent penetrates between the molecules, however, breaking the lateral linkages, the molecules may tend to curl up and so bring about a decrease in length of the fibre.

Various agents exist which break the several different types of lateral linkage; but because of the complex nature of proteins it is very rare that the molecules are held together by only one type of linkage, and the degree of response to any given reagent depends upon the relative proportions of the different types of linkage present.

If a fibrillar protein is heated in water, the thermal activity of the molecules will increase with rising temperature. If the protein is held together by weak linkages, increased thermal agitation may be sufficient to break some or all of the bonds, with resulting swelling or solution of the protein. Increased thermal activity is particularly effective in breaking Van der Waals linkages, but the solution of a protein in boiling distilled water does not mean, necessarily, that the protein is held together by these forces alone.

Vertebrate collagen first swells and then dissolves in boiling water. The amino-acid content of collagen is known with some degree of accuracy, and in view of the length of some of the side chains it is unlikely that linkages occur between the backbones. But it has been suggested (Jordan Lloyd and Garrod, 1946) that there are, on the average, two salt-links and one hydrogen bond between side chains to every ten residues. Thermal agitation suffices to break linkages of this sort as the temperature of the water increases.

A change in pH of the medium will affect the degree of ionization of the charged groups in the side chains and so tend to influence the formation of salt-links. Swelling or solution of fibrillar proteins in dilute acids or alkalis argues, therefore, for the presence of salt-links. If the protein is more soluble in acids than in alkalis, then there is a preponderance of basic side chains, and if more soluble in alkalis than acids, a preponderance of acid side chains.

Any factor increasing the dielectric constant of the medium will diminish the forces of attraction between polar groups, and de Bruyne (1939) has shown that, for wood, the swelling is approximately proportioned to the dielectric constant of the medium. Solution or swelling of electrovalent-linked proteins can sometimes be brought about, therefore, by treating them with liquids of high dielectric constant, such as formamide or urea solution; but these also have a specific effect on electrovalent links, due to the electrical polarity of their molecules.

Ions in solution may penetrate between the molecules and be absorbed at polar groups of opposite sign. The ions may act in two ways, by altering the dielectric constant of the medium or by neutralizing charges in the protein chain and hence control the amount of water that the protein structure contains. The effect of an ion is not only dependent on the size and charge of the ion, but is also influenced by the size of the water shell it carries with it. Calcium ions, which have a very small water shell, can approach very closely to negative groups and tend to form insoluble calcium salts. Therefore, calcium ions, though they break the electrovalent links, prevent solution of the protein. Lithium ions, which carry a large water shell, cannot approach sufficiently close to the negative groups to form salts, but come to lie between the negatively and positively charged groups, and concentrate about themselves the field of force. Under these conditions the protein still remains hydrated, and with the breaking of the electrovalent bonds may swell and go into solution. Whether the cation or anion is the effective ion in swelling a protein depends upon the isoelectric point of the protein. Strong solutions of lithium thiocyanate, for example, dissolve silk, in which the protein chains—composed largely of glycine and alanine (with very short side chains)—are held together mainly by linkages between the imino and carbonyl groups of the backbones.

The greater the number of linkages present the smaller is the chance that they will all be broken at one time, hence the smaller will be the effect of agents tending to break electrovalent bonds. For instance, the number of electrovalent bonds holding the structure together is increased by tanning collagen with vegetable tannins. Tanned collagen is no longer soluble in hot water and swells to a much smaller extent in dilute acids, alkalis, etc. Another example of the effect of the number of lateral linkages present on stability is the increasing solubility of cellulose in sodium hydroxide solution as the length of the cellulose chain is decreased.

The capacity of a swelling agent to influence the stability of a fibrous protein depends not only on the number and strength of the linkages present, but also on the spatial dimensions of the fibrous network. Nylon is held together by carbonyl-imino links between the backbone chains and readily dissolves in formic and thioglycollic acids and in m-cresol. Silk, which is also held together by carbonyl-imino links, is unaffected by thioglycollic acid and m-cresol. The failure of these two reagents to break the links in silk is believed to depend upon their relatively large molecular size, which makes it impossible for them to penetrate the compact silk-fibroin micells (Jordan Lloyd and Garrod, 1946).

Various methods of demonstrating the presence of electrovalent links in protein fibres can be devised in the light of these theoretical considerations, and from a study of the results it is sometimes possible to obtain an idea of the strength of the bonding. One of the factors which may make difficult the interpretation of the results is the presence in the structure of covalent links as well as electrovalent links.

To break covalent links it is necessary to employ specific chemical reactions. Keratin, for example, is held together by salt-links and disulphide bonds. If it is treated with alkali (which weakens the salt links) it can be stretched out in a way which suggests that some of the linkages holding the fibre-network rigid have been broken; but it is not possible to get keratin into solution by alkali treatment alone. If it is treated with sodium sulphide in alkaline solution or with thioglycollate solution, the reagent breaks the —S—S— bonds by reduction, and with both these and the salt-links broken, the keratin passes into solution (Goddard and Michaelis, 1934).

Slifer (1945) found that the chorion of grasshopper eggs was rapidly soluble in sodium hypochlorite solution. The chorion is composed of an aromatic tanned protein, and after testing all such known proteins with this solvent it has been found that they all dissolve in it very rapidly. The chemistry of the reaction is not known. The strength of the bond between the nitrogen and the benzene ring is approximately the same as that of the C—N bond in the backbone chains, so that solution probably occurs through breaking of both the peptide links and quinone bonds. The solution of these proteins has nothing in common with the solution of keratin in alkaline sodium sulphide which is preceded by swelling. In sodium hypochlorite the protein disintegrates, often with effervescence, and dissolves without swelling.

These two, the sulphide link and the quinone bond, are the only types of covalent links so far known to occur in biological skeletal proteins.

V. *Practical Details*

Structural proteins to be investigated are treated with the following reagents:

(1) Boiling distilled water.
(2) 0·2 N. Hydrochloric acid.
(3) 0·2 N. Sodium hydroxide solution.
(4) 6 M. Urea solution.
(5) Formamide.
(6) 2 M. Calcium chloride solution.
(7) Saturated lithium thiocyanate solution.
(8) Saturated calcium thiocyanate solution.
(9) 0·5 Thioglycollate solution (for method of preparations see Goddard and Michaelis, 1934).
(10) 10 per cent. Sodium hypochlorite solution.

Reagents 2 10 are used at room temperature and the experiments are continued for at least 24 hours before it is concluded that the result is negative.

Alteration in shape, or solution in boiling water indicates the presence of weak linkages easily broken by thermal agitation, while swelling or solution in dilute acids or alkalis, salt solutions, urea, and formamide indicate the presence of electrovalent linkages. Acids and alkalis attack salt-linkages in particular; acids most readily swell proteins with a high proportion of basic amino-acid residues; while those with a high proportion of acidic amino-acid residues are more readily swelled by alkalis. Hydrogen bonds and carboxyl-imono links tend to be broken by salt solutions and urea, but these reagents are not specific for these linkages.

Maximum swelling or swelling in thioglycollate solution indicates important —S—S— bonds; while solution only in sodium hypochlorite solution suggests the presence of aromatic tanning.

Chèvremont and Frédéric's (1943) method for demonstrating the presence of —SH and —S—S— groups in histological sections is useful for further confirming the presence of —S—S— bonds if the material can easily be sectioned. The demonstration of the presence of —S—S— bonds does not, however, mean that the protein is a keratin. Only those proteins which not only contain —S—S— bonds but also give a keratin-type X-ray diffraction photograph (Astbury, 1938) qualify for the title of keratins. There are other proteins which contain —S—S— bonds, but which give a collagen type of X-ray photograph; these should not be called keratins. Where it is impossible to obtain X-ray photographs, it is not possible to describe fully proteins containing —S—S— bonds.

When covalent links are formed between fibrillar proteins and aromatic tanning agents, the great stability of the system makes it difficult to devise tests to show conclusively the presence of the aromatic compound in the stabilized protein. Where tanning of this type occurs in structural proteins, however, there is good evidence that the precursor of the tanning agent is a polyphenol, which is subsequently oxidized to a quinone by a polyphenol oxidase (Pryor 1940; Stephenson, 1947; Dennell, 1949). Polyphenols are strong reducing agents and are able to react with certain reagents to give coloured compounds; both of these properties can be made use of to demonstrate the presence of polyphenols histochemically. When aromatic tanning is suspected, therefore, sections should be cut of the tissues responsible for secreting the structure, and these, rather than the structure itself, should be subjected to histochemical tests for polyphenols.

Orthodiphenols react with a very dilute solution of ferric chloride to produce a green colour turning to red on the addition of sodium bicarbonate. The chemistry of the reaction is not known. On treating thin sections in this way, the colour produced, even in the most favourable conditions, is extremely pale; but when it is possible to cut thick sections of fresh material by hand, this method is extremely useful, being highly specific for this type of polyphenol (Lison, 1936).

The reducing power of polyphenols has been used in the argentaffin reaction first described by Masson (for experimental details see Lison, 1936), which consists in the reduction of an ammoniacal silver nitrate solution leading to the deposition of silver at the site of reduction.

Diazonium hydroxides will react with phenols in tissue sections to produce coloured compounds, but they also react with histadine, purines, and pyrimidines (Lison, 1936; Mitchell, 1942; Danielli, 1947). It is possible, however, to treat the sections with reagents which will prevent one or more of these compounds reacting with the diazonium hydroxide. Differences in staining before and after such treatment make it possible to decide which of the substances listed above has been responsible for producing the colour (Mitchell, 1942; Danielli, 1947). If it is found, therefore, that a structural protein dissolves only in sodium hypochlorite solution, and is secreted by tissues containing a polyphenol, it may be concluded that there is circumstantial evidence for aromatic tanning.

Discussion

These methods have been used to investigate a variety of structural proteins in invertebrates and vertebrates, and the results of this investigation may briefly be summarized as follows:

While simple electrovalent linked proteins are of wide occurrence in *internal* supporting structures in both vertebrates and invertebrates, they are only used to form *external* membranes in aquatic organisms such as *Paramecium* and *Euglena* or in such terrestrial animals as earthworms (Goodrich, 1896) which are restricted to an environment of high relative humidity. In the Arthropods, the dominant group of terrestrial invertebrates, the protein component of the cuticle is hardened by quinone tanning and the whole cuticle is waterproofed by the addition of wax (Ramsay, 1935; Pryor, 1940; Wigglesworth, 1945; Beament, 1945). The external cortical layer of the cuticle of the intestinal parasite *Ascaris lumbricoides* (Nematoda) contains a quinone-tanned (Brown, 1950) —S—S— bonded (Chitwood, 1939) protein. Quinone tanning and —S—S— bonding also occur in the carapace of *Limulus* (Lafon, 1943); while —S—S— bonding occurs in the hooks and cuticle of Cestodes (Crusz, 1948). Quinone tanning has also been found in the central capsule membrane of the radiolarian *Thalassicola*, the chaetae of *Aphrodite*, the byssus and periostracum of *Mytilus edulis*, the byssus of *Dreissensia* (Brown, 1950), and in the egg cases of *Fasciola hepatica* (Stephenson, 1947) and of *Dendrocœlum* (Nurse, 1950). All these are *extracellular* proteins secreted by an underlying epidermis or gland. In the vertebrates, on the other hand, the subcutaneous structures are insulated from the outside world by —S—S— bonded *intracellular* proteins, the keratins, but quinone tanning has been found to occur in the *extracellular* proteins of the egg cases of Selachian fishes (Brown, 1950). Many structural proteins, however, still wait to be examined; it remains to ascertain what is the distribution of the various skeletal proteins in relation to phylogeny and to determine whether methods of stabilizing these other than those of electrovalent linkages, —S—S— bonding, and quinone tanning occur in the animal kingdom.

ACKNOWLEDGEMENT

I wish to acknowledge very considerable help from Dr. L. E. R. Picken and Dr. M. G. M. Pryor in the preparation of this paper.

REFERENCES

Astbury, W. T., 1938. Trans. Faraday Soc., **34**, 377.

Beament, J. W. L., 1945. J. exp. Biol., **21**, 115.

Brown, C. H., 1950. Nature, **165**, 275.

Chèvremont, M., and Frédéric, J., 1943. Arch. Biol. Paris, **54**, 589.

Chitwood, B. M., 1936. Proc. Helm. Soc. Wash., **3**, 39.

Crusz, H., 1948. J. Helminth., **22**, 179.

Danielli, J. F., 1947. Symposia of the Society for Experimental Biology, **1**, 101.

de Bruyne, N. A., 1939. Aircraft Engineering, **11**, No. 120, 44.

Dennell, R., 1949. Nature, **164**, 370.

Frey Wyssling, A., 1948. *Submicroscopic Morphology of Protoplasm and its Derivatives.* New York (Elsevier).

Goddard, D. R., and Michaelis, L., 1934. J. biol. Chem., **106**, 605.

Goodrich, E. S., 1896. Quart. J. micr. Sci., **39**, 51.

Jordan Lloyd, D., and Garrod, M., 1946. *Fibrous Proteins.* Leeds (Society Dyers and Colourists).

Lafon, M., 1943. Bull. Inst. Océanogr. Monaco, No. 850.

Lison, L., 1936. *Histochimie Animale.* Paris (Gauthier-Villars).

Meyer, K. H., 1942. *Natural and Synthetic High Polymers.* New York (Interscience).

Mitchell, J. S., 1942. Brit. J. exp. Path., **23**, 296.

Nurse, F. R., 1950. Nature, **165**, 570.

Pryor, M. G. M., 1940. Proc. Roy. Soc. B, **128**, 378.

——, Russell, P. B., and Todd, A. R., 1946. Biochem. J., **40**, 627.

Ramsay, J. A., 1935. J. exp. Biol., **12**, 373.

Slifer, E. H., 1945. Sci., **102**, 282.

Speakman, J. B., 1936. Nature, **138**, 327.

Stephenson, W., 1947. Parasit., **38**, 128.

Wigglesworth, V. B., 1945. J. exp. Biol., **21**, 97.

Methods for the Study of the Cytology of Bacteria and Pleuropneumonia-like Organisms

By EMMY KLIENEBERGER-NOBEL

(*From the Lister Institute, London*)

With one Plate

SUMMARY

Methods for the demonstration of the various cytological elements of bacteria a described. The specialized techniques applicable to pleuropneumonia-like organism and L-forms of bacteria are also presented.

DURING the last decade new methods for the study of various morpho logical features of bacteria and other minute organisms have bee developed including techniques for the demonstration of nuclear structure cytoplasm, cell walls, flagella, and capsular and slimy envelopes of bacteri It has been shown that bacteria may possess a life-cycle and may have definit stages which differ widely in appearance. For example, they may resembl pleuropneumonia-like organisms in their so-called L-form (Klieneberger Nobel, 1949). As an aid to students unfamiliar with the methods of morpho logical study of bacteria a number of fixing and staining procedures now i use have been collected below. These are preceded by a brief description o some forms of the bacterial cell as well as of pleuropneumonia-like organism

According to Robinow (1946) the usual bacterial cell consists of a cell wa lined with a cytoplasmic layer which may be slightly thicker at the two ce poles. The smallest bacterial cell has no transverse septum and no transvers cytoplasmic layer; it contains only one nuclear structure which, in a youn culture, may be on the point of dividing into two. With further developme the nuclear structure divides and the division-products move apart. The ce elongates at the same time, and a cytoplasmic septum is formed across th centre. With further growth a cell-wall septum may be formed which cuts th cytoplasmic septum into two layers and the cell into two compartments, eac of which may now contain one big nuclear structure or two smaller ones. A ce treated with a cytoplasmic stain is characterized by darkly stained cell tip (Pl. I, fig. 2); in preparations stained for nuclear structures the extreme tip are light (Pl. I, fig. 1). In older cells nuclear structures as well as cytoplasmi parts are often irregularly arranged. The shape, dimension, and arrangeme of the nuclear structures seem to bear a relationship to the changes whic bacterial cultures undergo. For example, in myxococci (Klieneberger-Nobe 1947*b*) the young cells are slender with two to four small, transversely arrange nuclear structures. The swarming cells possess two fatter nuclear bodie arranged centrally, leaving fairly large parts of the cell free of nuclear matte

[Quarterly Journal of Microscopical Science, Vol. 91, part 3, September 1950.]

The nuclear bodies of the swarming cells join together before the microcysts evelop. Nuclear fusion seems to be frequent in bacterial cells and often recedes spore formation (Klieneberger-Nobel, 1945). It can also occur as a hysiological reaction. When young cells are transferred to a lower temperaure a fusion may take place (Klieneberger-Nobel, 1947*c*). When anaerobes in young stage are exposed to the air the single nuclear structures collect in the nedian axis of the cell and combine to form a nuclear cylinder (Klieneberger-Nobel, 1945).

The location of the transverse septa may be of importance in the study of nicrobial development. Thus, for example, the sporulating hyphae of soil *Actinomycetes* (Klieneberger-Nobel, 1947*a*) possess very pronounced transerse septa while the primary or substratum mycelia have very delicate septa nly.

Furthermore, some species of bacteria possess mucoid envelopes which may e regarded as capsules proper or as amorphous slime (Klieneberger-Nobel, 1949). Flagella are generally difficult to stain; nevertheless, those of the o-called 'swarmers' in *Proteus vulgaris* are easy to stain.

When bacteria change from the A-form into the L-form (Klieneberger-Nobel, 1949), a marked transformation in their morphology takes place. In he cells of the L-form the nuclear material is often very finely dispersed and ell walls proper are absent (Pl. I, fig. 4). In consequence the individual units f the culture, which vary greatly in size and shape, are of a very fragile ature. Therefore special methods for the preparation of microscopical pecimens must be used.

The pleuropneumonia-like organisms are often composed of even more ragile elements than those of the L-growth of bacteria. Their colonies may be uilt up of small granules, fine flexible filaments, globular forms and flat, moeboid elements of various sizes (Klieneberger and Smiles, 1942). The lobules as well as the flat forms produce fine, darkly staining granules inside heir lumen which are regarded as the reproductive units and are able to nitiate new growth (Pl. I, fig. 5). The morphology of these organisms is tudied to advantage in special preparations carried out as described later.

METHODS

Coverslips, Slides, and Mounting of Preparations

For practical reasons the use of a coverslip is necessary in order to obtain ptimal resolution. The use of coverslips allows further all the necessary perations to be carried out in small containers such as watch-glasses nd small block dishes. This is very convenient and saves reagents and tains.

Stained coverslip films may be mounted in weak stain or in water. The reparation (avoid air bubbles) is gently pressed between layers of filter-paper nd then sealed with a rim of paraffin wax. The thinner the liquid layer etween coverslip and slide the better the resolution.

When preparations are mounted in Canada balsam after staining in wate solutions, they must be dehydrated. As a rule dehydration has to be carri out rapidly. For dehydration the following solutions used in histology a recommended:

1. Acetone, 19 ml.; xylene, 1 ml.
2. Acetone, 14 ml.; xylene, 6 ml.
3. Acetone, 6 ml.; xylene, 14 ml.
4. Two or three changes of xylene.
5. Canada balsam.

In some cases the staining qualities are improved by mounting in Cana balsam, but often the cells are slightly reduced by shrinkage which may be disadvantage.

Occasionally a neutral mounting medium which can be applied witho dehydration and which, therefore, does not produce shrinkage may be we come. The following medium, devised by Romeis and Wüst (Romeis, 193 has proved to be fairly satisfactory in some cases as, for example, in t mounting of stained encapsulated organisms:

Components

Gelatine.

Glycerine, twice distilled.

M/5 solution of borax (1·24 gm. borax crystals are dissolved in 10 m M-NaOH and brought up to 100; 0·1 ml. of a concentrated aqueo solution of $HgCl_2$ is then added).

Bromthymolblue solution (0·1 gm. bromthymolblue in 3·2 ml. M/20 NaO brought up to 250 ml. with distilled water).

M-NaOH solution.

Method

Seven gm. gelatine are added to 42 ml. of M/5 solution of borax. Place t gelatine in a hot-water bath until dissolved and add 42 ml. glycerine. Ac 10 drops of bromthymolblue solution to the warm glycerine-gelatine whi should become yellow. Now add drop by drop M-NaOH until the colo changes to blue. Filter the hot solution immediately (pH approximately 7· For use the glycerine-gelatine has to be liquefied by warming.

Preparation of the film

For the demonstration of details of bacterial structure it is of the greate importance to produce, whenever possible, a one-cell layer of organisms, w spaced and with each cell spread flatly on the surface of the coverslip. T best way of achieving this is to grow the organisms between a layer of sol medium and the coverslip. Clean, well-flamed coverslips (flame twenty tim quickly) are placed on sterile filter-paper disks in sterile Petri dishes. A pla containing the solid medium is inoculated evenly from a fresh culture means of a spreader. Inocula taken from a broth culture can be more evenly spread than those from cultures on solid media and will, therefore, yield more consistent results. Squares, smaller than the coverslips used, are cut from the freshly inoculated plate by means of a flamed knife or a Hagedorn needle. The squares are placed, inoculated side downwards, upon the coverslips. The Petri dishes containing the coverslip micro-cultures are incubated at the temperature required in a moist chamber which ought to close fairly tightly to allow for even humidity throughout. After growth has progressed to the stage desired, the micro-cultures are fixed and stained as described later. It is not necessary to grow the bacteria between agar and the coverslip for all purposes. When dealing with ordinary bacteria, good staining results may be obtained when the squares are cut from cultures already developed to the point desired. However, the coverslip should not be pressed down on the medium and lifted up, a manipulation which involves gross displacement; a cut-out piece placed cautiously on the coverslip is always preferable. Yet it should be realized that when dealing with soft and fragile material and when very fine detail of structure is desired, the micro-culture is the best method.

Only sparsely grown cultures will yield the required one-cell layer. Yet the periphery of colonies or the growth developing on the coverslip in the humid zone round the edge of the agar square often supply suitable material for the study of older growth. The development in this zone may be encouraged by running a drop of liquid round the edge of the freshly inoculated square or by using a medium of soft consistency. It must be realized that suitable growth on the coverslip of the organism under observation is of greater importance than the fixing and staining methods applied later and that each new subject studied involves a little ingenuity to ensure the necessary flat, thin, and well-spaced growth on the coverslip.

Fixing and Staining

A. *Chromatinic Structures*

Osmium tetroxide/Giemsa method. Remove the agar square with the tip of a knife while holding the coverslip with a pair of forceps. Take care that the piece does not slip along the coverslip. Put the coverslip with adherent film quickly into osmium tetroxide vapour. Use for fixation a small glass jar with ground-in, greased lid. Fill it with glass beads up to three-quarters of its height. Pour in 5 ml. of a 2 per cent. aqueous osmium tetroxide solution, best prepared from crystals obtainable in sealed ampoules containing 1/10 gr. each. The jar must be refilled every fortnight or more often if much used. Fix the wet film in the vapour (film side upwards) for 3–5 minutes. Allow to dry in the air.

Cells of organisms poor in ribonucleic acid can be stained with Giemsa solution alone (Gurr's R 66). Use two or three drops of Giemsa solution in 3–5 ml. of a mixture of tap-water and freshly distilled water. Place the coverslip film-side downwards into the Giemsa solution to prevent precipitation on the surface of the specimen. For example, certain stages of the vegetative forms of *myxococci* and some water organisms such as *Sphaerotilus natans* stain well by this simple method, showing up red chromatinic structures in a bluish cytoplasm. Also very young cells of ordinary bacteria and L-forms can often be stained in this manner quite satisfactorily. Mount in a drop of stain without washing or dehydrate and mount in Canada balsam.

Osmium tetroxide / Hydrochloric acid / Giemsa method (*Robinow*). Most bacterial organisms, however, possess a cytoplasm very rich in ribonucleic acid. Consequently they have a strong affinity for all basic dyes by which they are stained so deeply that the nuclear structures are obscured. The ribonucleic acid is gradually decomposed when the fixed coverglass films are immersed in normal HCl (film side up) at a temperature of approximately 55° C. The time of this treatment varies with the species and the stage of the organism. For example, 4 minutes seem to be the correct time for 3-hour agar cultures of *B. coli* or *Proteus vulgaris*. After treatment the films are rinsed in water and placed in Giemsa solution (as above) and mounted as described. The periods of staining must be determined by trial: 20 minutes should suffice for the above-mentioned young cultures. Usually the nuclear structures appear deep red or black, and the cytoplasm takes a bluish colour. If applied too long, the hydrochloric acid produces an unstainable cytoplasm, yet the desoxypentose-nucleic acid contained in the chromatinic structures is very resistant to the action of the acid. The staining method described above was first devised by Piekarski and introduced into bacteriology and widely used by Robinow.

Osmium tetroxide / Ribonuclease / Giemsa method (*Boivin, modified*). After fixing and drying as before, the coverslips are placed film side up in a watch-glass standing in a warm place (37° C. incubator or on top of water-bath). The film is covered with a 0·1 per cent. solution of ribonuclease which is allowed to remain for 4–20 minutes or longer according to the object. It is then rinsed in water, stained with Giemsa and mounted as before. The effect of ribonuclease is similar to but slower than that of hydrochloric acid, but the staining of the cells often shows a particularly beautiful contrast between the red nuclear structures and the blue cytoplasm. The ribonuclease has no effect on cells which are difficult to penetrate, such as spores, while the hydrochloric acid can be used for these as well as for vegetative cells. The ribonuclease treatment first introduced by Boivin has been widely used by him and his collaborators. However, Boivin used it in combination with a particular fixation method outlined below.

Chabaud/Ribonuclease/Giemsa method (*Boivin*). Place the dried coverslip film into Chabaud's fixative (alcohol 80 per cent., 60 ml.; formalin, 5 ml.; glacial acetic acid, 2 ml.; phenol crystals, 15 gm.) for 10 minutes. Rinse in water, apply ribonuclease, stain and mount as before. Preparations made in this way show good staining contrast, but the fixative produces shrinkages of the cells, which may be of serious disadvantage when dealing with delicate elements of structure.

Osmium tetroxide/Giemsa/Eosin (*Badian*). Fix with the vapour of osmium tetroxide solution as above, stain with Giemsa solution (20 minutes are sufficient for *B. coli* and *Proteus vulgaris*). Mix two drops of a 1 per cent. watery eosin solution with 5 ml. distilled water in a watch-glass. Put the stained coverslip, unrinsed, into the eosin solution and leave for 30 seconds, rinse and mount in water. This simple method often produces good contrasts, but like the ribonuclease method has no effect on spores.

It should be mentioned that the Feulgen reaction, if applied in a suitable way, demonstrates clearly the chromatinic structures of bacteria, yet the cell as a whole remains unstained.

Pl. I, fig. 1, shows the regularly arranged chromatinic structures of a spore-bearing Gram-positive bacillus stained by the hydrochloric acid/Giemsa method. Pl. I, fig. 4, shows a water vibrio in the L-stage stained by the hydrochloric acid/Giemsa method.

B. *Cytoplasm* (*Klieneberger-Nobel, Robinow*)

Use either micro-cultures or squares cut out from plate cultures and placed on coverslips. Place the coverslip with adherent agar square upwards into a watch-glass and cover with Bouin's fixing solution (sat. solution of picric acid, 20 ml.; formalin, 10 ml.; glacial acetic acid, 1 ml.). Leave for 1½ hours; remove the agar square. The bacterial film should adhere firmly to the surface of the coverslip. Wash well, stain in Giemsa solution for 30 minutes or as required. Mount in the dilute stain. The cytoplasmic areas, tips of the cells, cytoplasmic layers, and septa are stained bluish or purple. The areas of the cells occupied by the chromatinic structures remain unstained.

Pl. I, fig. 2, shows a culture of *B. coli* with its cytoplasmic areas well stained.

C. *Cell Walls and Transverse Septa* (*Robinow*)

Fix the wet film with the vapour of osmium tetroxide solution or use Bouin's solution through the agar as described above. Place the preparation (film side downwards) into a 5 per cent. tannic acid solution for 20–30 minutes. Wash thoroughly under the tap and place the coverslip in a weak solution of crystal violet for a few seconds or longer as required (two drops of a 0·5 per cent. watery solution of crystal violet with 5 ml. of distilled water). Mount in a drop of stain. The tannic acid mordant renders the interior of the cells practically unstainable while the walls and the transverse septa are distinctly stained. Pl. I, fig. 7, shows cells of a *Proactinomyces* stained in the manner described.

D. *Flagella of* Proteus *sp.* (*Klieneberger-Nobel*)

Pour an agar plate and dry it in the incubator for several hours in an inverted position with the lid ajar. Place a small inoculum of *Proteus* in the middle and leave the plate at warm room temperature for approximately 24 hours, by which time a swarming edge should have developed. Watch for motility under the low power of the microscope. If motility is observed, cut out agar squares, half the surface of each of which is covered with growth. Place the squares on coverslips and fix with Bouin's solution as described before. Wash and stain with Giemsa solution for approximately 18 hours. Mount in a drop of the stain or dehydrate and mount in Canada balsam. Pl. I, fig. 3, shows swarming cells of *Proteus* sp. stained for flagella.

E. *Capsules* (*Klieneberger-Nobel*)

Use a nutrient agar containing 30 per cent. of horse serum. Plant the organism to be examined on the surface of the plate and incubate in a CO_2 atmosphere (about 10 per cent.) for a few hours or until a delicate layer of growth is visible. In particular cases it may be of advantage to add carbohydrate to the medium. Cut out squares, put them on coverslips and fix with Bouin's solution as above. Wash, place in Giemsa for several hours, and then mount in the stain. The organisms should stain darkly, the capsules should stain a delicate blue or pink. A nice contrast may be obtained if staining with Giemsa is followed by counterstaining with a thin eosin solution (see above). Leave in the eosin for a few seconds only, wash and mount in water.

If tannic acid is applied for 30 minutes after the fixation and the film stained in crystal violet for about 10 minutes the capsules stain a deep violet while the organism remains unstained. Though giving occasionally very good contrast this method is not consistently successful.

It should be mentioned that the capsule staining methods described depend on the serum content of the culture medium. Probably any serum would serve the purpose though horse serum has been used here. Pl. I, fig. 6, shows organisms of the *Klebsiella* group prepared by the Bouin/Giemsa method.

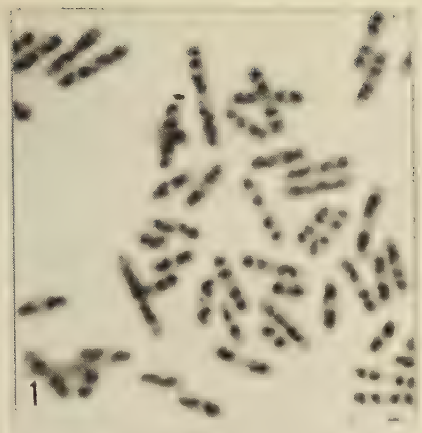
F. *Slime* (*Klieneberger-Nobel*)

Mix a loop of the cultures to be examined and a loop of saline and spread it on the coverslip. Dry in air and place into Chabaud's solution (see above) for 10 minutes. Rinse and place in 5 per cent. tannic acid or in Löffler's mordant for 30 minutes. Wash thoroughly and stain in crystal violet for several hours. Mount in the stain or in water. The organisms should be unstained with dark contours; the amorphous slime should be stained pink.

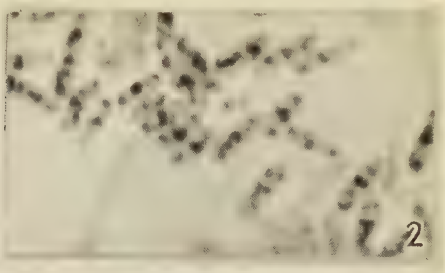
While the capsules of all organisms examined could be well demonstrated by means of the methods described, the slime of some organisms stained distinctly while in the case of others the staining was not so successful.

G. *Pleuropneumonia-like organisms and L-forms of bacteria*

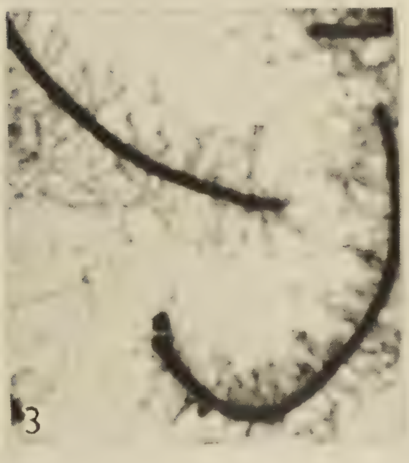
For the study of pleuropneumonia-like organisms, micro-cultures are prepared from suitable medium. (From ox heart infusion-peptone agar, a boiled blood medium according to Levinthal (1921) is prepared. This is filtered under sterile conditions. Sterile horse serum is added.) The micro-cultures are incubated in a moist chamber. They are fixed at appropriate times with Bouin's solution, washed and stained in weak Giemsa solution for several hours or, better, overnight. They can be dehydrated to advantage in the acetone-xylene mixtures mentioned above and mounted in Canada balsam.



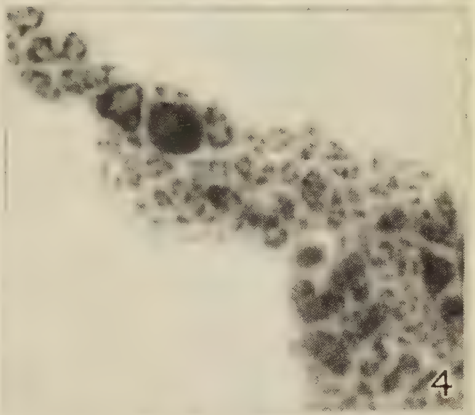
1



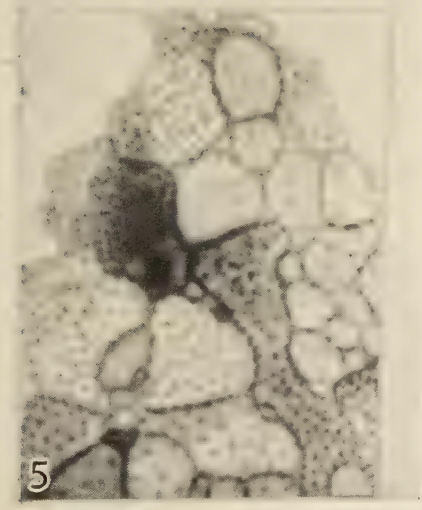
2



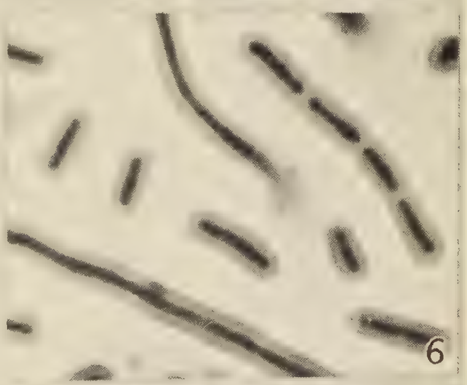
3



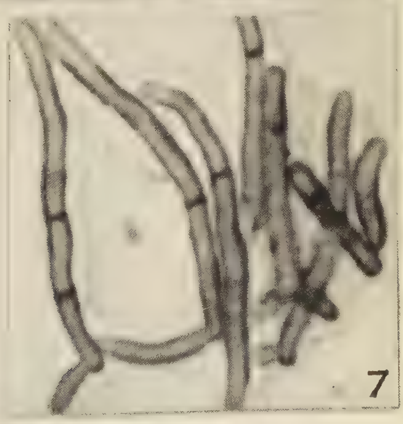
4



5



6



7

E. KLIENEBERGER-NOBEL—PLATE I

Care should be taken that dehydration is carried out as rapidly as possible. It is advisable to blot the water off gently before the actual dehydration. In preparations made as described no appreciable dislocation of elements should have occurred. They should show whole stained colonies, the delicate edges of which can be examined under the high power of the microscope. Bodies containing granules are usually developed after about 48 hours of incubation. The vapour of osmium tetroxide also fixes pleuropneumonia-like organisms, but owing to their extreme delicacy they do not stand up well to the separation of the agar square from the coverslip, which has to precede fixation. L-growth is often more resistant. It usually withstands the separation process, particularly if it is not pure but interspersed with bacillary forms. Therefore osmium tetroxide fixation can be used for the fixation of the L-growth. This fixation may be followed by the hydrochloric acid treatment and by staining with Giemsa solution. Pl. I, fig. 5, shows the 2-day-old growth of the pleuropneumonia-like organism isolated from bronchopneumonic lungs of rats. The relatively large bodies are filled with fine filterable granules. The preparation was carried out by the Bouin/Giemsa method. The L-growth of the water vibrio of Pl. I, fig. 4, was demonstrated by the osmium tetroxide/hydrochloric acid/Giemsa method.

REFERENCES

BADIAN, J., 1933. Arch. Mikr., **4**, 409.

BOIVIN, A., 1948. Revue médicale de Liége, **3**, 237.

KLIENEBERGER-NOBEL, E., 1945. J. Hyg. Camb., **44**, 99.

—— 1947*a*. J. gen. Microbiol., **1**, 22.

—— 1947*b*. Ibid., **1**, 33.

—— 1949. Ibid., **3**, 434.

KLIENEBERGER, E., and SMILES, J., 1942. J. Hyg., **42**, 110.

LEVINTHAL, W., 1921. Ergebn. allg. Path. path. Anat. 19 Jahrg., II. Abt., **5**, 878.

ROBINOW, C. F., 1942. Proc. Roy. Soc. B, **130**, 299.

—— 1944. J. Hyg., **43**, 413.

—— 1946. Addendum in Dubos, R. J., *The Bacterial Cell.* Harvard (University Press).

ROMEIS, B., 1932. *Taschenbuch der mikroskopischen Technik*, 13. Aufl., München und Berlin (Oldenburg).

EXPLANATION OF PLATE I

(Magn. × 2,500–3,000)

FIG. 1. Gram-positive bacillus, young. Hydrochloric acid, Giemsa. (Chromatinic structures.)

FIG. 2. *Bact. coli*, young. Bouin, Giemsa. (Cytoplasm.)

FIG. 3. *Proteus* sp., swarming organisms. Bouin, Giemsa. (Flagella.)

FIG. 4. Water vibrio growing completely in the L-phase. Hydrochloric acid, Giemsa. (Chromatinic structures.)

FIG. 5. Pleuropneumonia-like organism from bronchopneumonic lungs of rats. Bouin, Giemsa prolonged. (Granules in large bodies.)

FIG. 6. Organism of Klebsiella group. Capsule staining method 1.

FIG. 7. *Proactinomyces.* Bouin, tannic acid, crystal violet. (Transverse septa.)

On the Use of Wide Illuminating Cones in the Microscope

By A. G. OETTLÉ, *Nuffield Travelling Medical Fellow*

(*From the Department of Human Anatomy, Oxford*)

SUMMARY

In bright-ground illumination, glare is noticeable when the condenser aperture is equivalent to that of the objective. It can still be detected, though to a greatly reduced extent, after most causes of glare have been avoided. It is suggested that this residual glare is inevitable, and represents the loss of contrast resulting from a relative excess of direct rays, which occurs when the direct light falls in the marginal zone of the objective, since half of the diffracted rays then falls outside the lens.

ON theoretical grounds it has generally been assumed that the optimum conditions for bright-ground illumination will be attained when the numerical aperture of the illuminating cone equals that of the objective. None the less, it is just as well known that in practice better contrasts are obtained in the images of absorbing objects if the aperture of the condenser be reduced a trifle below that of the objective. This is not a question of resolving power, but of contrast, and all authors agree that unless the utmost resolution is necessary, the condenser aperture should be less than that of the objective. Authorities differ on the question how much less, some advocating a two-thirds cone (Martin and Johnson, 1931), others a three-quarters (Nelson, 1910), and others a nine-tenths cone (Belling, 1930; Shillaber, 1944).

REASONS PREVIOUSLY SUGGESTED TO EXPLAIN THE IMPROVEMENT PRODUCED BY A REDUCED CONDENSER APERTURE

Although some writers have been at a loss to explain why the image should improve, the usual explanation is that the outer zones of the objective are less well corrected than the central region. While this may be true in some instances, and is easy to demonstrate in the presence of gross spherical aberration in the objective, it does not fully explain what is observed with well-corrected objectives. If the trouble were due to zonal aberrations in these lenses, the performance should improve still further if the objective as well as the condenser were stopped down, so that the diffracted as well as the direct rays were excluded from marginal zones. This is not the case.

The question was considered in 1922 by Beck, who demonstrated that the use of wide illuminating cones was associated with excessive glare, which could be controlled by a field stop that would allow no more than one-fifth of the area of view of a ×25 eyepiece. Further improvement might also be obtained by immersion contact between objective and the slide, thus abolishing the reflectivity of the upper surface of the coverslip. Beck states: 'I am

[Quarterly Journal of Microscopical Science, Vol. 91, part 3, September 1950.]

inclined to think that if the corrections of the aberrations of a lens are perfect and the area of the object is not larger than one-fifth the field of view with a ×25 eyepiece, no appreciable glare will be produced whatever the angle of the illumination.' If one cannot use the field stop, then it will be necessary to stop down the condenser. Shillaber (1944, p. 393) has pointed out that closing the condenser diaphragm is a means of reducing glare whatever the cause, although this manœuvre is obviously undesirable as it leads to loss of resolving power and makes prominent the diffraction lines around image detail.

While agreeing with Beck's observations on the benefit that accrues from confining the illumination to a small area of the slide—and this seems to be the main advantage of the well-corrected condenser system—the writer believes that the question has not been completely settled. Even when all possible precautions have been taken to avoid glare, an improvement in contrast, slight but distinct, can still be observed when the aperture of the condenser is reduced below that of the objective. This fact was commented on by Rheinberg (1929), who arrived at essentially the same explanation as that presented in this paper. Since he only touched in passing on this subject, his work appears to have been almost entirely overlooked; and as he presented no experimental evidence in support of his thesis, it seems desirable once more to draw attention to this subject.

EXPERIMENTAL EVIDENCE

A Leitz achromatic objective of N.A. 1·32 was used as a condenser, and a similar lens, with a built-in iris diaphragm, as objective. Köhler's method of illumination was used, the condenser was accurately centred, and axial illumination obtained. A dark-green filter (Wratten 62) was employed, together with a well-focused field stop. Unfortunately the aperture stop in the objective did not coincide with the image of the condenser iris diaphragm, but the two could be seen superimposed by removing the eyepiece and viewing through a pinhole cap on the tube. The system was checked for absence of spherical aberration. As an object, a stained blood-smear, mounted in balsam between two coverslips, was chosen, since this introduces no problems of critical resolution and does not possess too high an intrinsic contrast. It was oiled to both the objective and the condenser. In this way glare from most causes can be avoided apart from that arising by reflections at the different components of the objective.

The images produced with condenser apertures equal to those of the objective were found to be considerably better than one would have expected, and, as Beck has already observed, these did not deteriorate on opening the aperture of the condenser wider still. (According to Zernike (1942), under these conditions the image resembles that of a self-luminous object, as Lord Rayleigh indicated in 1896 (p. 242); Conrady (1904) denied that this could happen.) None the less, a slight improvement in contrast could still be produced by stopping down the condenser aperture below that of the objective. As this was done, the amount of light in the background was reduced, and a faint haze that had been covering the edges of the cells disappeared. The image continued to improve until the condenser aperture was reduced to about three-quarters of that of the objective. If, at the point of optimal contrast, the diaphragm in the objective were closed until the aperture of the objective was reduced to that of the condenser, the faint haze returned, only to disappear once more if the condenser aperture were reduced a second time. This could be repeated until the reduction in numerical aperture in the objective produced too great a loss in the resolution and the image itself became blurred.

It appears from these experiments that this type of image deterioration occurs when direct light falls in the marginal zone of the available aperture of the objective. To see if this were so, a series of annular condenser stops was employed with the same objective. For convenience, a Cooke phase-contrast condenser system was used since this provides a series of annuli which can be accurately centred. It was unfortunate that the aperture of the hollow cone given by the largest of these annuli was not much more than 0·7, and the objective (N.A. 1·32) needed to be stopped down considerably to equal the condenser aperture. Furthermore, with the lamp available, a very small field stop could not be used since it acted as an aperture stop as well, i.e. the rays did not diverge sufficiently after passing through this stop to illuminate the whole annulus. The obvious solution—to place a ground glass screen behind the field stop—was not adopted since this would reduce the amount of light available as well as being likely to increase glare.

With the objective at full aperture (N.A. 1·32), annular illumination, provided by the largest of the annuli, gave satisfactory images. As the aperture of the objective was reduced, image deterioration could be noticed, but contrast did not alter significantly until the aperture of the objective stop as seen at the back of the objective was less than one-third as great again as that of the annulus. The background now appeared to brighten, and on further closure of the diaphragm, contrast became poorer. In the field of view a circle of haze appeared and contracted concentrically with the field as the aperture was reduced, finally forming a bright central spot from which a dark expanding circle appeared with a further closure. In this dark area the cells appeared in oblique dark-ground illumination, and the objective stop could be shown to be smaller than the illuminating annulus. (This observation will probably be familiar to those who use an objective fitted with an iris diaphragm for dark-ground studies.) If, at the point where image contrast begins to fall off, one changes to a smaller annulus, a return to improved contrast can be observed. The effects seen here are thus not attributable to the annular illumination, nor to reduction of aperture *per se*, but to the zone of the objective illuminated by the direct rays, contrast being poorest with marginal illumination.

The effects described have been observed with objectives of all types and powers, though it has not always been possible to control glare as carefully as in the experiments quoted.

Explanation

It cannot be denied that what has been observed might be due merely to the effects of slight amounts of glare that could not be avoided in the experiments, but, none the less, Rheinberg's explanation seems at least as feasible. The third possibility, that the improvement observed is due to the beneficial effect of closing the condenser iris on phase detail, need not be considered, since the phase change introduced by blood-cells in balsam is far too small to be visible at this aperture (Oettlé, 1950).

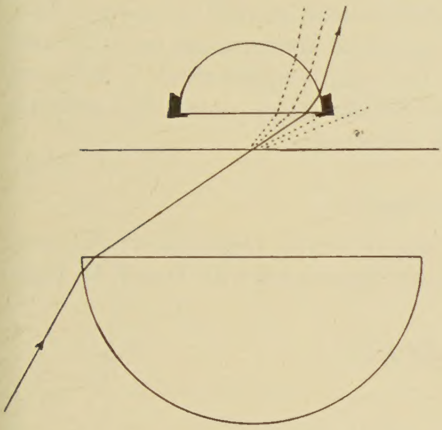


Fig. 1

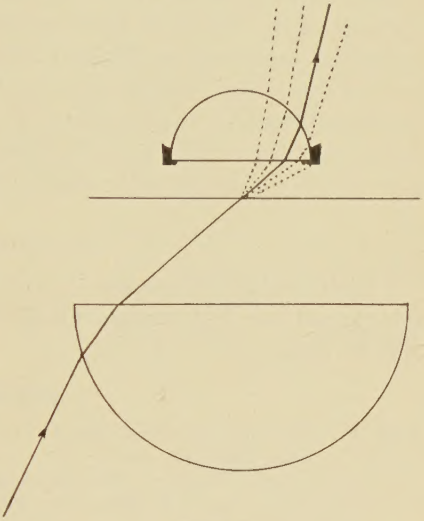


Fig. 2

Text-fig. 1. Diagram of the back lens of the condenser and the front lens of the objective. A marginal ray is drawn to show the loss of half of the light arising by diffraction at the object. The diffracted rays are indicated by broken lines.

Text-fig. 2. This depicts a ray passing through a zone internal to the marginal zone, showing that more of the diffracted light enters the objective.

According to the simple form of the Abbe theory, the microscopical image corresponds to the object when the entire diffraction pattern is gathered by the objective. Text-fig. 1 shows that when the direct rays strike the marginal zone of the objective one-half of the diffraction pattern is automatically excluded from image formation. There will therefore be a relative excess of direct light as compared with diffracted light, which will manifest itself in the image as a deterioration in contrast. When the aperture of the rays is reduced below that of the objective a greater proportion of diffracted light will enter the objective (Text-fig. 2), and contrast will consequently be better.

This deterioration in contrast resulting from the loss of diffracted light was visualized by Abbe in 1889, though it led him to the conclusion that for perfect similarity of image and object an exceedingly narrow axial cone of illumination would be preferable to a wide cone—an opinion that ran counter to the experience of practical microscopists (Nelson, 1910) who have been vindicated on theoretical grounds by Conrady (1904, 1905) and more recently by Zernike (1942, 1943).

This explanation of image improvement on closing the condenser iris diaphragm below that of the objective is not intended to supplant the reasons put forward by Beck in 1922. It is presented as additional to his explanations. It is hoped that it will serve to allay any unrest like that displayed by Wredden (p. 75), at having to depart in practice from the 'ideal'. Certain other facts fit in well enough with this presentation: for example, it explains why the annulus in the phase-contrast microscope should be well below the maximum aperture of the objective. Here too lies the reason, in all probability, why the technique suggested by Wells in 1875 and Schulze in 1878 of using a darkground condenser with an objective of equivalent aperture has never become popular. Finally, it may explain why so few microscopists think it necessary to immerse the condenser with high aperture objectives, unless it is essential that the maximum possible resolution be obtained.

ACKNOWLEDGEMENTS

I wish to thank Professor le Gros Clark, in whose Department this work was carried out, and to acknowledge helpful discussions with Drs. J. R. Baker and R. Barer.

REFERENCES

Abbe, E., 1889. J.R. micr. Soc., **9,** 721.
Beck, C., 1922. Ibid., **42,** 399.
Belling, J., 1930. *The Use of the Microscope.* New York (McGraw Hill).
Conrady, A. E., 1904. J.R. micr. Soc., **24,** 610.
—— 1905. Ibid., **25,** 541.
Martin, L. C., and Johnson, B. K., 1931. *Practical Microscopy.* London (Blackie).
Nelson, E. M., 1910. J.R. micr. Soc., **30,** 282.
Oettlé, A. G., 1950. Ibid. (in the press).
Rayleigh, Lord, 1896. Phil. Mag., **62,** 167. Scientific Papers, **4,** 242.
Rheinberg, J., 1929. J. R. micr. Soc. **49,** 132.
Schulze, A., 1878. J.R. micr. Soc., **1,** 45.
Shillaber, C. P., 1944. *Photomicrography in Theory and Practice.* New York (Wiley).
Wells, S., 1875. Boston J. Chem., 140, quoted by Woodward, 1878.
Woodward, J. J., 1878. J.R. micr. Soc., **1,** 246.
Wredden, J. H., 1947. *The Microscope, its Theory and Applications.* London (Churchill).
Zernike, F., 1942. Ned. T. Natuurk., **9,** 357.
—— 1943. Handelingen van het XXIXe Nederlandsch Natuur -en Geneeskundig Congres, gehouden op 27, 28, en 29 April 1943 te Amsterdam.

A few

BRAND MICHROME BRAND

STAINS & REAGENTS

FOR MICROSCOPY

Acridine Orange
Adonitol
Alizarin Red
Aniline Blue-Orange G
Arabinose
Auramine, O
Azo Carmine, B & G
Azo Eosin
Azur 1, 2, A, B, C, & L
Azur 2 Eosin
Bromcresol Green
B.Z.L. Blue
Canada Balsam
Carminic Acid
Cedarwood Oil
Celloidin
Chlorazol Black
Cresyl Fast Violet
D.P.X. Mountant
Dulcitol
Eosin, Azur
Erythrosin
Evans Blue
Fast Green, F.C.F.
Field's Stain
Gallamine Blue
Gallocyanin
Giemsa Stain
Haematein
Haematoxylin
Indian Ink, Micro
Indigo Carmine
Indophenol
Janus Black
Janus Blue
Janus Green, B
Janus Red
Jenner Stain
Leishman Stain
Light Green-Clove Oil
Maltose
May-Grunwald Stain
Metachrome Yellow 2RD
Methyl Green
Methyl Green-Pyronin
Methylene Violet (Berthsen)
Naphthol Green
Neutral Red
Night Blue
Nile Blue
Osmic Acid
Panchrome (Pappenheim)
Peptones
Phloroglucinol
Pontamine Sky Blue
Purpurin
Resazurin
Rose Bengale
Rosolic Acid
Ruthenium Red
Saffron
Safranin
Salicin
Sodium Taurocholate
Sudan Black
Sudan Blue
Sudan Brown
Sudan, 1, 2, 3, & 4
Tartarzine
Thioflavin, T
Titan Yellow
Toluidine Blue
Trypan Blue
Trypsin, Bact.
Victoria Blue
Vital New Red
Wright's Blood Stain
&c.

Water Analysis Tablets. Low Viscosity Nitrocellulose for embedding
Water-soluble Indicators. Stains in tablets and ampoules
Slides and Cover Glasses: Laboratory Sundries

A FEW OF THE NEW PRODUCTS PREPARED ONLY BY EDWARD GURR, LTD.

Borrel Methylene Blue Powder. For easy and rapid preparation of Borrel Methylene Blue solution.

Cedronol. The superfine cedarwood oil for immersion lenses. Xylol miscible. Refractive index 1·520.

Ehrlich Haematoxylin Powder. For easy and rapid preparation of Ehrlich Haematoxylin solution, ready for immediate use, without ripening.

Optoil. A xylol miscible, synthetic, non-sticky and non-drying immersion oil. Refractive index 1·515.

For SERVICE, UNIFORM HIGH QUALITY, RELIABILITY and for IMMEDIATE DELIVERY Stipulate

BRAND MICHROME BRAND

Prepared only by

EDWARD GURR, LTD.

108 Waterford Rd., Walham Green, LONDON, S.W.6

Price List and Literature on application

Telephone : Renown 2011 *Cables :* Gurr, Renown 2011 London

Now ready: **'MICROSCOPIC STAINING TECHNIQUES'**

No. 1, price 1s. 6d. (24 pages); No. 2, price 3s. 6d. (62 pages)

The Quarterly Journal of MICROSCOPICAL SCIENCE

THE SUBSCRIPTION for Vol. 91 is £3. 3s. 0d. for the Four Numbers; for this sum (*prepaid*) the JOURNAL is sent Post Free to any part of the world. Separate numbers and back numbers which remain in print are sold at 21s. net each.

At the CLARENDON PRESS, OXFORD, *or*
OXFORD UNIVERSITY PRESS, LONDON, E.C. 4

NOTICE TO CONTRIBUTORS

CONTRIBUTIONS TO THE JOURNAL should be addressed to the Editors, Q.J.M.S., and sent to Dr. C. F. A. Pantin, Dept. of Zoology, Downing St., Cambridge. If any preliminary communication on the subject of a paper offered to the Editors has already been published in another journal, a note to this effect must accompany the manuscript.

Manuscripts must be typewritten (double spaced). Typescripts should be fully corrected; an allowance of 10*s*. per sheet of sixteen pages is made for alterations in proof, contributors being responsible for any excess.

Manuscripts should be as concise as possible. Papers not exceeding 6,000 words can receive most rapid publication. Extensive introductory and descriptive matter commonly required in theses for university degrees should **not** be included in the manuscript.

Words to be printed in italics should be underlined. Division of the matter into subheadings must follow a consistent plan and excessive subdivision must be avoided.

All manuscripts must be accompanied by a **summary**, not exceeding 5 per cent. of the length of the paper and in no case exceeding 500 words. The summary should contain no illustrations or tables and should be intelligible by itself without reference to illustrations or tables in the body of the paper. It will be printed in small type immediately after the title of the paper.

Footnotes should not be used.

Acknowledgements should be placed at the end of the paper, before the references.

References should be given in the following style:

Lison, L., 1936. *Histochimie Animale*. Paris (Gauthier-Villars).

Wigglesworth, V. B., 1933. Quart. J. micr. Sci., **76,** 270.

If it is considered desirable to give the titles of papers, they should be placed in inverted commas immediately after the dates of the references.

Illustration should not be redundant. Authors may be asked to provide a grant for excessive illustration. Illustrations (including lettering) should be arranged so that they may be printed as figures of a size appropriate to a page of the journal. The maximum size of a printed figure is $5'' \times 7\frac{1}{2}'' = 13$ cm. × 19 cm.

Illustrations should be made about half as large again as the size required in print. Thus a drawing destined to occupy a whole page should be about $7\frac{1}{2}'' \times 11''$ (20 cm. × 30 cm.), including lettering.

Drawings should be 'line and dot' in black process ink. (The attention of authors is drawn to 'A Method of Illustration for Zoological Papers', by H. G. Cannon, published by the Association of British Zoologists, 1936.)

Photographs should not be given if a line and dot drawing will serve the purpose. Photographic prints should be sharp and with good contrast.

Lithographic and coloured illustrations will **not** be published unless the author is prepared to make a grant covering the cost of their production.

Position of Figures and Plates should be clearly indicated by notes written in the margin of the typescript.

The lettering of figures should be written in block capitals on accurately imposed outlines on tracing-paper. Abbreviations should not be used unless absolutely necessary. These remarks apply both to text-figures and to plates.

The magnification of a figure should be indicated by a scale drawn on it.

Authors receive 50 copies of their contributions gratis, and may buy additional copies if they apply to the Editors when they return the corrected proofs.

Bodies of numerical data too extensive for complete publication should be summarized for discussion, and the originals deposited in the Archives established for this purpose in the British Museum (Natural History).